













# **APPROVED LABORATORY TECHNIC**

**CLINICAL PATHOLOGICAL, BACTERIOLOGICAL,  
MYCOLOGICAL, VIROLOGICAL, PARASITOLOGICAL, SEROLOGICAL,  
BIOCHEMICAL AND HISTOLOGICAL**

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# APPROVED LABORATORY TECHNIC

## GENERAL LABORATORY METHODS

### THE MICROSCOPE AND METHODS OF MICROSCOPY

#### EQUIPMENT

The microscope is one of the most essential and most frequently used instruments in the laboratory. An instrument of recent manufacture is always desirable. There is no choice among microscopes insofar as the optical systems are concerned, but one may prefer one or the other type of standard design. Their general construction is shown in Figure 1. They should be handled and carried only by the arm H in order not to put a strain on the coarse and fine adjusting mechanisms. The *monocular* types of microscopes are more commonly used because they are least expensive. The *binocular* microscopes, however, are more desirable because they induce less fatigue over longer periods.

The *optical system* of a microscope consists of (1) the objective or lens which is compound, *i.e.*, made of a series of lenses; (2) the ocular or eyepiece which further magnifies the image, and (3) the substage condenser which concentrates light on the object as it rests upon the stage, thereby increasing the illumination.

**Objectives.**—Objectives are named by their equivalent focal length; also according to their construction or manner of use, as (1) dry objectives, (2) immersion objectives, (3) achromatic objectives, and (4) apochromatic objectives.

**DRY OBJECTIVES.**—These are simple with air alone between the front lens and the coverglass of the preparation.

**IMMERSION OBJECTIVES.**—Some are so constructed that water must be placed between the front lens and the coverglass for the objective to function properly. Others require more illumination than can be delivered by the condenser with air space or water between lens and objective. Cedar wood oil, which has the same refractive index as glass, must be placed between the front lens and the coverglass and between the glass slide and the condenser. These are the well-known oil immersion objectives. They will not permit full illumination or clear resolution without being immersed in oil. The common practice of using paraffin oil because it is less sticky is not recommended.

**ACHROMATIC OBJECTIVES.**—These are objectives in which the image is free from the rainbow colors. A simple or single lens does not bring light of different wave lengths (different colors) to a common focal point. The light of shortest wave length (the blue-violet end of the spectrum) is bent or refracted most by a lens and comes to a focus nearer the lens than light of a longer wave length (red end of the spectrum) which rays are bent or refracted less. This means that the image of a colored object will

not be in sharp focus for all colors. The phenomenon is called *chromatic aberration*. It may be corrected by placing a second, concave, flint glass lens behind the primary convex, crown glass lens. The use of fluorite or fluorspar in the second lens permits a higher degree of correction. Achromatic objectives are corrected for two colors. They are also corrected at the same time for *spherical aberration*.

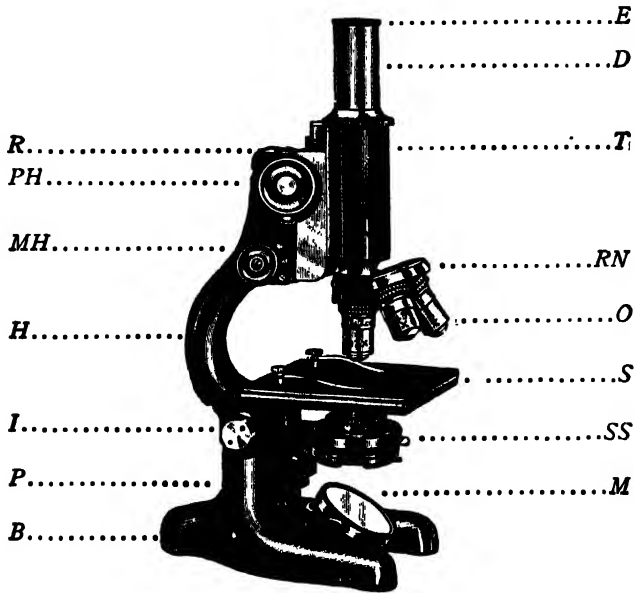


FIG. 1.—A SATISFACTORY TYPE OF MICROSCOPE

*E* is the eyepiece, of which two or three are usually furnished for varying degrees of magnification.

*D* is the draw tube, which is calibrated and should always be drawn to 160 or any other length recommended by the manufacturer.

*T* is the body tube.

*RN* is the revolving or triple nosepiece carrying the objectives.

*O* is one of the usual three objectives.

*R* is the rack upon which the tube is raised or lowered.

*PH* is the pinion screw for coarse adjustment.

*MH* is the micrometer screw for fine adjustment.

*H* is the handle.

*S* is the stage.

*SS* is the substage carrying the Abbé condenser with diaphragm.

*M* is the mirror with plane and concave surfaces.

*I* is the inclination joint for using the microscope in an inclined position.

*P* is the pillar.

*B* is the base, which should be large and solid.

**APOCHROMATIC OBJECTIVES.**—These are corrected for spherical aberration and for three colors and are, therefore, more desirable for photographic purposes.

As a general rule there should be three objectives on a triple, revolving nosepiece, namely, a 16 mm., a 4 mm. and a 1.9 mm. (oil immersion).

**Oculars.**—Oculars are usually designated by their magnifying power, as 5X, 7.5X, 10X, etc. It is their function to pick up the image formed by the objective and enlarge it further. Thus the degree of magnification of an object visualized may be determined

by multiplying the magnification power of the ocular by the magnification number of the objective. (Tube length must be properly adjusted.) Oculars are also designated according to their construction. *Huygenian oculars* are the least expensive and those most commonly used. They do not possess the correction of better forms for color or flatness of field. *Compensating oculars* are overcorrected so as to further reduce chromatic and spherical aberration of an objective. They should always be used in conjunction with apochromatic objectives.

**Magnification.**—The magnification of any combination of objectives and oculars may be obtained by multiplying the magnification of the objective by that of the ocular. The magnification given by different combinations of objectives and oculars is as follows:

Objectives	Oculars		
	6.4 ×	10 ×	
16 mm. (10 ×)	× 64	× 100	
4 mm. (43 ×)	× 275	× 430	
1.9 mm. (95 ×)	× 610	× 950	

**Substage Condensers.**—These are constructed usually of two lenses for the purpose of concentrating light upon the object as it rests upon the stage of the microscope. They thereby increase the illumination. They are commonly of the nonachromatic type named after their designer Abbe. Aplanatic, achromatic condensers are available on the more expensive microscopes.

**Illumination.**—Daylight from a north window is the ideal source of illumination. However, it is so often unavailable that a more constant and dependable source is desired. Many forms of microscopic lamps are offered by manufacturers (see Figs. 2, 3 and 4). The substage lamp or any lamp utilizing a 100 watt nitrogen filled tungsten bulb and a "daylight" glass filter provides a satisfactory source of light. A powerful source, such as the carbon arc or 6 volt ribbon filament bulb is required for darkfield illumination.

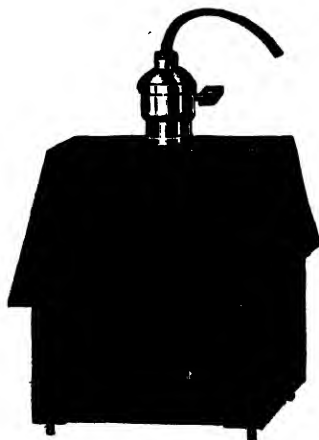


FIG. 2.—MICRO LAMP, CHALET FORM

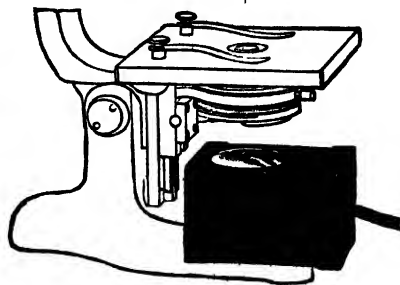


FIG. 3.—MICRO LAMP, SUBSTAGE FORM



FIG. 4.—MICRO LAMP. FOR 100 WATT NITROGEN FILLED BULB OR RIBBON FILAMENT BULB  
(6 volt)

When equipped with the latter, the lamp is suitable for darkfield illumination (Bausch and Lomb).

**Accessory Equipment.**—A mechanical stage is advisable for blood counting or wherever a systematic search of an object is required. It is also well to be provided with a *hand lens* for the study of tissues (stained slides), sputa and feces, etc., prior to microscopic study. It gives one an idea of the composition of the whole specimen and enables one to better orient one's self when studying the smaller fields seen through the objective. In the selection of material from sputa or feces better samples will be obtained by the aid of such a lens. A pointer in the ocular is a great convenience. If the top of the lens of the ocular is removed, a diaphragm with a circular aperture will be found in the ocular tube. An eyelash or any fine hair may be so cemented on the rim of this diaphragm by means of Canada balsam so that the free end of the hair is in the center of the aperture. With the top lens in place the hair should appear as a fine pointer of focus. If it is not in focus, move the diaphragm up or down until the hair is seen in sharp definition. This will serve to locate objects in the field. For the enumeration of small objects, like blood platelets or reticulocytes, it is helpful to cut down the size of the field. This may be accomplished by cutting a disk of black paper or metal to such a size that it will fit snugly over the diaphragm in the ocular. Cut a small square about 5 or 6 mm. on a side in the center of this disk. It can be removed easily when it is not needed.

### THE USE OF THE MICROSCOPE

1. The microscope should rest upon a table or desk of such height that when one is seated before it one can comfortably look into the ocular without inclining the instrument. If seated before an open window or any other source of light the eyes should

be so shaded that no other rays of light enter the eye but those from the microscope. If using a monocular form, both eyes should be kept open and one should learn to relax the accommodation of the unused eye. The ability to do this may be acquired by means of a black card. An opening is cut in a 3 x 6 inch card near the center of one narrow end, large enough to fit snugly over the upper end of the draw tube, after the ocular has been removed. The card then projects into the field of vision of the unused eye. If the surface is blackened it reflects little or no light into that eye. After a time the card may be discarded.

2. *The tube length should be adjusted.* The draw tubes of most microscopes are graduated so that the tube may be pulled out to the proper length for each objective. The tube length for which each objective is corrected is engraved upon it. For most lenses this length is 160 mm. Tube length is extremely important with oil immersion objectives. A variation of 5 mm. will destroy the perfection of the image. Increased or diminished tube length alters the initial magnification of the objective. The tube should be withdrawn with a spiral motion while supporting the coarse adjustment screw lest the whole body tube be drawn from its bearing. It is returned to its former position with the same care lest the objective be driven forcibly against the stage or object.

3. The microscope lamp should be placed about 10 or 12 inches from the mirror and its rays directed upon the mirror (plane surface). Swing the condenser out of position or rack it down to its lowest point. Look into the tube at the back of the low power (16 mm.) objective and manipulate the mirror with both hands until the tube is evenly illuminated. The ocular is now replaced and the object to be examined is placed upon the stage.

4. Focus the low power objective by first viewing it from the side and placing it down near (within 1 or 2 millimeters) the object. Then, looking into the ocular, rack the body tube upward by the coarse adjustment screw until the object comes into sharp focus. Illumination is now further adjusted by setting the condenser at the lowest point which gives even illumination yet brilliant sharp definition of the object. It is well to examine the objects always with the lower powers first as directed above, selecting fields to be studied with the higher magnification. Once the light is centered it need not be changed, but the position of the condenser must be changed for each objective and for each variety of object studied. It must be higher for dense objects and quite low for unstained or transparent objects such as urinary casts. The illumination just described is called *central illumination*. It is the type most frequently used. After a little experience one may place objective and condenser in their approximate positions and adjust the mirror without removing the ocular. The centering is finally determined by focusing up and down upon the object. If the illumination is correctly centered, the image moves up and down, in and out of focus. If not correctly centered the image will move to one side or another and back again as the objective is raised or lowered. Where the sharpest definition is desired, the aperture of the condenser must also be considered for each objective. After correct centering of the light and focusing of the condenser and objective, the ocular is removed and one looks at the back of the rear lens of the objective. Slowly the iris diaphragm of the condenser is closed until its shadow is seen in the periphery of the back lens.

With oil immersion objectives on bacteria the iris should be wide open. Where surface markings are to be studied, as on diatoms, cut the apparent aperture to about



$\frac{2}{3}$  and in still denser objects, such as histologic preparations, to about  $\frac{1}{2}$  the opening of the back lens. With dry objectives the iris should be cut down to slightly less than the aperture of the objective so that its edges should just be visible in the periphery of the back of the lens.

With the oil immersion objective the condenser must be racked upward to its highest point. Oil should be placed between the condenser and the slide and between the coverglass and the objective. Focusing, with this and all objectives, should be *upward* after placing the lens near the object. *Never* focus down upon an object. The fine adjustment screw is used to study the object after it has been brought into focus by the coarse adjustment. The fine adjustment screw should never be turned more than one complete revolution in either direction. It is operated best by the left hand and constantly adjusted as the right hand manipulates the mechanical stage or the slide upon the stage.

In the study of surface contour, *oblique illumination* may be necessary. Research microscopes are so arranged that the iris diaphragm can be shifted from the center to the periphery condenser. Without this arrangement one must use a finger or a card over the aperture of the condenser so that the light may enter it from one side only. In this manner one may demonstrate the cylindrical shape of urinary casts.

Aside from these suggestions the adjustment of the condenser and the iris diaphragm become a matter of experiment in each case, in order to determine the best sort of illumination to bring out the desired details in the object to be studied.

5. In order to obtain the best illumination for objectives more than 16 mm. E.F. the condenser should be removed and the plane or curved mirror used—depending upon the degree of illumination desired. When the condenser is used with objectives of 16 mm. E.F. or less (the high powers) the plane mirror should always be employed. The condenser is so constructed that parallel rays of light are brought to a focus above the uppermost surface of the top lens of the condenser and in the plane of the object. If the concave mirror is employed, the rays of light are brought to a focus within the condenser and its effectiveness is depreciated. One may use the concave mirror and the condenser for the lower powers, instead of removing the condenser entirely, and obtain satisfactory illumination of the field. Under such circumstances the condenser must be moved close to the object.

**Coverglass Thickness.**—A coverglass should be used with wet preparations. It is not so important when using the lower power objectives, but even here the roundness of the drop formed causes a distorted field that is not so easily studied. A coverglass must be used with the higher power objectives lest the objective be wet by the liquid and the image be distorted and the lens of the objective be soiled. Covers are made of 3 standard thicknesses classified as No. 1, No. 2 and No. 3. From the optical standpoint No. 2 is best suited for all but oil immersion objectives. For those, No. 1 is necessary because of the short working distance (space between objective and object). Even the best grades vary considerably in thickness and the variation affects the distinctness of the image. With the low powers this effect is so slight that it may be ignored, but with the 4 mm. objective a deviation of 0.05 mm. above or below the standard for which the objective is corrected may obliterate fine structures in the image. One may avoid this difficulty by selecting covers of the proper thickness with a micrometer or by the use of the adjusting collar with which some objectives are fitted. The thickness of coverglasses is about as follows:

No. 1 .....	0.13 to 0.17 mm.
No. 2 .....	0.17 to 0.25 mm.
No. 3 .....	0.25 to 0.50 mm.

Slight variations in coverglass thickness may be compensated for by increasing the tube length for covers too thin or decreasing it for covers too thick. With a 4 mm. objective of 0.85 numerical aperture, an increase of 30 mm. in tube length will balance a decrease in coverglass thickness of 0.03 mm. It must be borne in mind that a change of tube length changes the initial magnification of the objective.

When employing oil immersion objectives, cover thickness is not so important, provided the combined thickness of it and the mounting medium is less than the working distance of the lens. Tube length is of considerable importance with immersion objectives, however, for a difference of 5 mm. is sufficient to destroy the perfection of the image.

### THE CARE OF THE MICROSCOPE

The microscope should be covered at all times when not in use. A close mesh, lintless cloth boot that covers the entire stand is quite efficient. A glass bell jar or a transparent cellulose jar is convenient. If the glass jar is used, great care must be exercised in placing it over the microscope lest it strike and damage the instrument.

Any accumulation of dust should be wiped from the stand with a soft cloth kept for that purpose. The cloth moistened with xylol will remove cedar oil. Alcohol should never be used on any part of the microscope. The coarse adjustment, rack and pinion should be cleaned from time to time as the lubricating oil tends to become thick and sticky. Remove the body tube from the stand, after racking it up as far as it will go, and wipe the rack and pinion screw with a cloth moistened with xylol, until all dirt is removed. The parts should then be wiped with a clean cloth moistened with a good grade of paraffin oil and the tube replaced. The bearings of the mechanical stage may be cleaned and lubricated in the same manner. The coarse adjustment screw may need tightening from time to time. The method differs with each make of microscope. Consult the book of instructions which comes with the microscope. No other adjustments should be made except by an expert microscope mechanic. The agents for the manufacturer will gladly make such minor adjustments without charge. It is well to have the microscope inspected and adjusted occasionally by such an expert so that the life of the instrument will be prolonged and its efficiency maintained.

The lenses should be brushed frequently with a soft camel's hair brush. Then they may be wiped and cleaned with a soft cloth (well washed linen) or lens paper. If the cloth or paper is used before brushing, the gritty particles always found in dust will scratch the lens surface. Oil may be removed from the immersion lens with a piece of lens paper moistened with xylol. The lens system must never be taken apart. The surface of front and back lens may be cleaned as directed above, but never separated except by an expert microscope mechanic.

### MICROMETRY

A good micrometer is a practical necessity as the importance of size in identification of microscopic structures cannot be emphasized too strongly. Even very rough measurements will often prevent humiliating blunders. The principal microscopic objects which

are measured clinically are bacteria, animal parasites and their ova, and blood corpuscles. The metric system is used almost exclusively. For very small objects, 0.001 millimeter has been adopted as the unit of measurement, under the name *micron*. It is represented by the Greek letter  $\mu$ . For larger objects, where exact measurement is not essential, the diameter of a red blood corpuscle (7 to 8 micra) is sometimes taken as a unit. Of the several methods of measurement, the most convenient and accurate is the use of a micrometer eyepiece (Fig. 5). In its simplest form this is similar to an ordinary eyepiece, but it has within it a glass disk upon which is ruled a graduated scale. When this eyepiece is placed in the tube of the microscope, the ruled lines appear in the microscopic field and the size of an object is readily determined in *terms of the divisions of this scale*. The value of these divisions in millimeters manifestly varies with different magnifications. Their value must, therefore, be determined separately for each objective. This is accomplished through use of a stage micrometer



FIG. 5.—MICROMETER EYEPIECE WITH MOVABLE SCALE

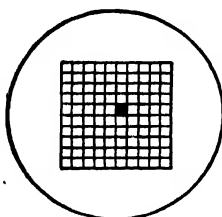


FIG. 6.—OCULAR MICROMETER DISK

—a glass slide with a carefully ruled scale divided into subdivisions, usually hundredths of a millimeter. The stage micrometer is placed upon the stage of the microscope and brought into focus. The tube of the microscope is then pushed in or pulled out until 2 lines of the one scale exactly coincide with 2 lines of the other. From the number of divisions of the eyepiece scale, which then correspond to each division of the stage micrometer, the value of the former in micra or in frac-

tions of a millimeter is easily calculated. *This value, of course, holds good only for the objective and the tube length with which it was found.* The counting slide of the hemacytometer will answer in place of a stage micrometer, the lines which form the sides of the small squares used in counting red blood corpuscles being 50 micra apart. When using the counting chamber with an oil-immersion lens a cover must be used, otherwise the oil will fill the ruled lines and cause them to disappear. Any eyepiece can be converted into a micrometer eyepiece by placing a micrometer disk — a small circular glass plate with ruled scale, ruled side down — upon its diaphragm (Fig. 6). If the lines upon this are at all hazy the disk has probably been inserted upside down or else the diaphragm is out of its proper position. Usually it can be pushed up or down as required.

### DARKFIELD MICROSCOPY

In the preceding pages, bright field microscopy has been discussed wherein objects have been studied microscopically by transmitted light, that is to say, light from the condenser passed through the object and was modified by the natural color or artificial stain of the object. Those objects which possess little or no differential coloring of their various parts are stained by selective dyes to cause them to stand out prominently. Thus the nuclei of cells are rendered visible in the cytoplasm, and bacteria barely visible are made quite prominent. Often it is desirable to study living things that cannot be easily stained or would be destroyed by a dye and are yet so tiny that even with the oil immersion lens they would be invisible by transmitted light.

As one gazes about the ordinary room no dust particles are seen by the naked eye, but if one directs the line of vision at right angles to a beam of sunlight, entering through a window, myriads of tiny particles become apparent because they reflect the rays of sunlight into the eye. One sees them not because the light is brighter but because they reflect the sun's rays into the eye. The phenomenon is spoken of as the "Tyndall effect". By the same principle, nonluminous stars or the moon are seen in the sky at night because of the sun's rays which they reflect to the earth. This is the principle of darkfield illumination. No light enters the microscope except that reflected by the objects in the field. Hence, the background is dark and the objects appear bright. Illumination may be from above the stage or from below it. When illuminated from below the effect may be obtained with the lower power objectives by placing a metal disk supplied with many microscopes beneath the iris diaphragm of the Abbe condenser. This shuts out the central rays and allows only the peripheral border rays to enter.

When higher magnification is required, as in searching for *Treponema pallidum*, greater obliquity of light is necessary because of the increased numerical aperture of these objectives. One of the regularly designed dark-field illuminators must be employed (Figs. 7, 8). One may use one of the combined types. These are refracting condensers of the Abbe type fitted with interchangeable lens mounts. The advantage of such a condenser is that one may shift from bright field illumination to darkfield by lowering the condenser and changing the top element.

This type is not a very satisfactory darkfield illuminator. The paraboloid illuminator is the most practical for routine work. It is easily manipulated and gives an excellent darkfield. The cardioid illuminator provides light rays of even greater obliquity and, therefore, can be used with objectives of higher numerical aperture with all of its advantages. The result is a brighter object with a much darker background and greater resolving power. However, the cardioid illuminator is much more difficult to manipulate, and it is much more sensitive to dust particles or scratches upon the slide or coverglass. The paraboloid type may be used as follows:

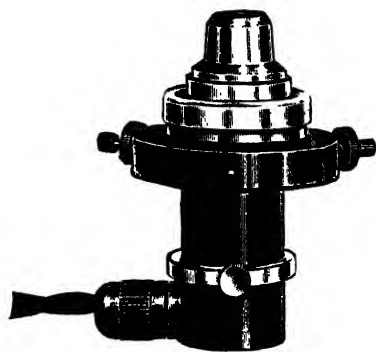


FIG. 8.—A DARKFIELD CONDENSER WITH LIGHT ATTACHED

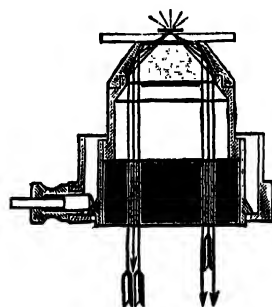


FIG. 7.—SECTIONAL VIEW OF PARABOLOID DARKFIELD CONDENSER

1. The oil-immersion lens has too great a numerical aperture for this work. It must be cut down by placing a funnel stop behind the rear lens. The proper stop is furnished by the maker of the objective. It should provide a numerical aperture of 0.80. The threaded end of the objective is unscrewed and the stop placed in position, apex down in the lens mount. The threaded end is then screwed back in place and the objective is screwed into the nosepiece.

2. The Abbe condenser is removed from its substage adjusting sleeve and the

darkfield illuminator fastened in its place. One must make certain that the upper lens surface is in the plane of the upper surface of the stage or a little higher when the illuminator is racked to its highest point. Close contact with the under surface of the glass slide may then be assured. The diaphragm of the illuminator should be wide open.

3. The source of illumination, a carbon arc lamp or a ribbon filament lamp, should be placed about 12 inches from the mirror and a narrow beam of light directed on the plane mirror. The beam should nearly fill the mirror surface.

4. As the light from the lamp is reflected up into the illuminator, some of it is reflected by the polished under surface of the illuminator back into the mirror. This light may be reflected on the wall behind the lamp or on the lamp housing as a very much dimmer light spot than that which comes directly from the light source. These 2 spots of light may be easily seen if the mirror is manipulated. The mirror will be correctly placed if the dim spot reflected from the illuminator can be directed back into the light source through the front lens of the lamp. The low power objective is focused on the upper surface of the condenser before placing the preparation on the stage. When some light passes into the illuminator even though the illumination is not uniform, a small circle will be seen, scratched upon the surface of the top lens. This indicates the center of the lens and by means of the centering screws on the illuminator mount, this small circle must be brought into the exact center of the field.

5. One is now ready to prepare the material for examination. Glass slides of a definite thickness must be selected. The correct thickness is usually indicated upon the illuminator mount. The Bausch and Lomb illuminator is adjusted for slides of not greater than 1.55 mm. If thicker slides are used, the illuminator cannot be brought into proper relation with the object. If the slide is too thin, the illuminator may be racked down to the proper level. The coverglass must not be more than 0.18 mm. thick (No. 1). The material to be examined must be in a thin emulsion. Too many particles will cause too much scattering of light and diminish the sharpness of the field. The scraping or juice expressed from chancres in searching for *treponema pallidum* must not contain too much debris, pus or blood. It is well to practice making suspensions of varying density with salt solution. Scrapings from the margins of the gums will provide excellent material with which to practice.

6. Raise the illuminator so that its upper surface is in the same plane as the surface of the stage. Place a drop of immersion oil upon the illuminator lens and place the preparation in the oil in position. Examine the preparation with the 16 mm. objective. If the condenser is in proper position, a circle of light will appear in the center of the field. This spot is more easily seen if a ground glass slide of exactly the same thickness as the one used for the preparation is substituted, ground side placed upwards (Fig. 9). (The ground glass slide which should be a part of the equipment can be made by rubbing a slide of proper thickness in a paste made of water and very fine emery or carborundum powder on a piece of plate glass.) If the illuminator is too high or too low, a ring of light with a dark center will be seen. Raise or lower the illuminator until a circle is obtained. If this is not possible, the slide is too thick or the illuminator is not properly set in its holder. If the illumination appears to be correct, swing the immersion lens into position and, after placing a drop of immersion oil upon the cover, bring the objective into focus (focus upward). The background should be dark and the scattered bodies in the field should be bril-

liantly illuminated. Slight adjustments of the height of the condenser and the position of the mirror will improve the blackness of the background and the brilliance of the illuminated bodies. Finally, when the best setting is obtained slowly close the iris diaphragm until there appears the most contrast of brightness of object and blackness of field. As in the bright field microscope exact centering of the illuminator will be

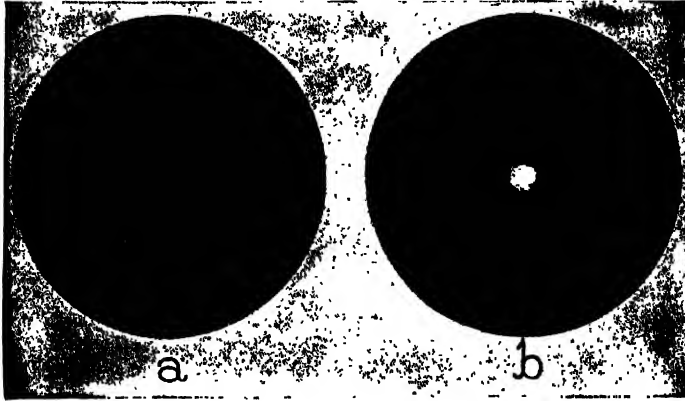


FIG. 9.—DARKFIELD ILLUMINATION

- (a) Improper illumination because the condenser is above or below the correct focus.  
(b) Proper illumination with the bright spot when the condenser is correctly focused. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

evidenced by an up and down movement of the image as the fine adjustment screw raises or lowers the objective in and out of focus. A lateral motion of the objects in the field indicates that the illuminator is not centered.

Sources of failure in darkfield illumination are: (a) Insufficient illumination; (b) condenser out of focus or decentered; (c) glass slide too thick; (d) failure to close iris diaphragm or to use funnel stop of correct N.A. and (e) preparation too dense.

# METHODS FOR THE CARE, INOCULATION AND BLEEDING OF ANIMALS AND DIAGNOSIS OF ANIMAL DISEASES

**Principles.**—1. Suitable animals are indispensable for certain laboratory procedures, as follows:

(a) For the detection of tubercle bacilli in sputum, urine and other materials; for the detection of tetanus and other anaerobic bacilli in wound discharges; for the detection of glanders, anthrax, tularemia and other organisms in various discharges and materials; for securing a rapid growth of pneumococci for type differentiation in relation to the serum treatment of pneumonia; for testing the virulence of diphtheria bacilli in relation to the lifting of quarantine in diphtheria and for raising the virulence of some pathogenic organisms, notably streptococci and pneumococci.

(b) For aiding the isolation of certain organisms such as tubercle and glanders bacilli, pneumococci, etc.

(c) For the preparation of vaccines and the propagation of certain viruses that cannot be cultivated artificially, such as in smallpox and rabies.

(d) For preparing sera for diagnostic serological tests like complement fixation, agglutinins, precipitins and various hemolysins.

(e) For testing the curative activity and toxicity of various chemical agents like arsphenamine and other organic arsenicals employed in the treatment of syphilis and other diseases, sulfonamide compounds, etc.

(f) For the preparation of various immune sera employed in the prophylaxis and treatment of diphtheria, tetanus, meningococcus meningitis, gangrene, pneumonia, etc.

(g) For testing the antibody strength of diphtheria, tetanus, pneumococcus and other immune sera.

2. Animals should be healthy, selected with care, and provided with comfortable and clean housing as well as sufficient and appropriate food.

3. Few of the methods of injection and bleeding produce any more pain than similar procedures in human beings, but all major operative procedures should be conducted under full anesthesia and all animals at all times should be handled and treated with the tender care merited by their great service to humanity, as well as to the lower animals themselves.

## HOUSING ANIMALS

1. The cages for small animals such as rabbits, guinea-pigs, mice and rats should be so constructed and arranged that the animals are kept dry, comfortable and sanitary. Overcrowding should be avoided. Separate cages should be provided for normal unused animals.

2. Animals from outside sources should be carefully inspected for evidence of disease before being placed in the cages for normal animals. If space permits, the new animals should be quarantined for a week or 10 days.

3. The cages should be constructed to permit thorough cleansing and disinfection and arranged for sufficient light and ventilation. Various models are available from supply houses or may be built to meet special requirements.

4. Special isolated quarters or cages should be provided for animals inoculated with very infectious material and especially that known or suspected of containing

*B. anthracis*, *B. mallei* (*Pfeifferella mallei*), *B. tularensis* (*Pasteurella tularensis*), rabies, hog cholera, foot and mouth disease, etc.

5. Inoculated animals should be kept alone in cages to guard them from annoyance or even injury by normal animals.

6. The animal quarters should be well lighted and ventilated. A uniform temperature during the entire day and night should be provided.

### IDENTIFICATION OF ANIMALS

A dependable system of identification of the animals should be adopted. The practice of cage labeling is suitable for stock animals kept in large groups, although in the interest of complete records each animal should be numbered and registered with regard to sex, description, etc.

1. **Tagging.**—For rabbits and guinea-pigs a small aluminum tag may be used. It is held in place by small staples passed through the eyelets of the tag and then through the ear and the ends bent over.

For larger animals such as horses, cows, pigs, etc., there are various types of tags which are supplied with special instruments for attaching. These may be obtained through veterinary supply houses.

2. **Banding.**—This method of identification is employed chiefly for fowl. A band bearing the number is fastened around the leg. There are several types, commonly called "leg bands", which may be purchased from poultry supply houses.

3. **Description.**—If this method of identification is employed alone, animals of different markings or color should always be selected if more than one is to be placed in a cage. Rubber stamp drawings of the mouse, rat, guinea-pig and rabbit may be purchased to facilitate these records. This method is usually used in conjunction with the tagging method to insure proper identification should the tag be lost. Any deformities or peculiar markings should also be noted carefully. The sex should be recorded as a part of the description (male ♂ and female ♀).

4. **Marking.**—Small animals like mice and rats may be marked by coloring the hair and skin with dyes (saturated alcoholic solutions of fuchsin or picric acid) on the body or along the tail. Cages should be labeled.

### FEEDING OF ANIMALS

Stock diets which are ordinarily fed to laboratory animals may be inadequate for nutritional requirements.<sup>1</sup> This is particularly true in relation to several of the vitamins.<sup>7</sup> Even moderate deficiencies or variations in rations may markedly affect laboratory animals. Rations supplied in pellet form by the Arcady Farms Milling Company, Chicago, Ill., may be recommended for rabbits, guinea-pigs, white rats and mice.

1. **Mice.**—Mice may receive stale bread soaked in water or skimmed milk. A small amount of cod liver oil may be added once a week (approximately 1 ounce for 300 mice). Crushed barley or rolled oats or moistened middling may be given at regular intervals. An excellent ration recommended by Keeler consists of rolled oats (240 parts), powdered skim milk (30 parts), cod liver oil (8 parts), and salt (1 part). The formula for rat feed prepared by Maynard<sup>3</sup> is equally satisfactory. Greens in



the form of lettuce or clover should be given occasionally. Drinking water must be available at all times.

2. **Rats.**—One may use either the Maynard standard diet or prepare a mash consisting of boiled beans, wheat maize meal, cabbage, and cod liver oil. Beef or liver and fresh cabbage should be offered once a week. A mixture of boiled vegetables supplemented by oats, corn, or white bread mixed with milk is equally satisfactory. Drinking water should always be available.

3. **Guinea-pigs and Rabbits.**—Rolled oats or crushed barley, bran and a good quality of alfalfa or clover hay represent the basic ration which must be supplemented with green feed, either cabbage or carrots, and beets. From time to time, salt, fish meal, and boiled potatoes may be offered. If the food contains considerable moisture, no water need be supplied. Spontaneous scurvy is by no means uncommon among guinea-pigs maintained on a diet which lacks green stuffs.

Aside from a careful selection of freshly prepared food mixtures, or wholesome vegetables and greens freed from tainted or rotten spots, it is important that a regular system of feeding be adhered to strictly. All animals should be fed and cared for once a day, including Sunday. A double ration of food thrown into the cages on Saturday will not supply the required nourishment on Sunday.

### CLINICAL OBSERVATIONS OF INOCULATED ANIMALS

1. Inspection once, or preferably twice, daily for *general appearance* should be given with a view of detecting symptoms. Each animal should be observed in motion; the consumption of food and water, and the appearance of the feces should be noted. Attention should also be paid to possible salivation, nasal and conjunctival discharges, and to the reactions at the site of inoculation.

**TABLE 1.—NORMAL TEMPERATURE, PULSE, AND RESPIRATION  
OF EXPERIMENTAL ANIMALS**

Animal	Average Rectal Temperature °C. and °F.	Pulse Rate	Respiration Per Minute
Guinea-pig	38.6° C.; 39.4° C.—7.5 cm. from anus (101.5° F.—4 cm. from anus) (Minimum 37.8° C., maximum 40.5° C.)	150	100-150
Rabbit	39.6° C. (102.4° F.) (Minimum 38.3° C., maximum 40.8° C.) No temperature under 40.0° C. should be considered pathologic)	120-140	50-60
Rat	37.9° C. (100.0° F.)	—	210
Mouse	37.4° C. (99.3° F.)	120	—

2. The *weight* should be recorded before experiment and afterwards at weekly intervals or more often, as the circumstances require. Each weighing should be done as nearly as possible under the same conditions as the first one.

3. The *temperature* should be taken in many cases before beginning the particular experiment and subsequently on each successive day at the same hour. For the sake of convenient reference, the normal average temperature is given in Table 1 in order to prevent the erroneous assumption that a pyrexia is present in an animal which shows merely its own normal temperature.

4. A *hematologic study* is frequently indicated. The general principles are the same as customarily used in the diagnostic laboratory. The normal averages of the different blood cells are summarized on page 52.

### SELECTION OF ANIMALS FOR DIAGNOSTIC TESTS

1. **Staphylococcus Infections.**—The pathogenicity of a staphylococcus should be studied on the rabbit or on Japanese white mice. Significant lesions may be observed following intravenous injections, while local reactions may be induced by scarification of the cornea. Toxic metabolic products may be tested by intravenous injection or by intradermal application.

2. **Streptococcus Infections.**—There is some difference of opinion as to which animal is most susceptible. If relative sizes of animals are considered, the rabbit is the most readily attacked. Various methods of infection are chosen in order to imitate the pathologic process from which the coccus has been isolated. Virulence tests are frequently made on mice. Great care should be exercised in the interpretations of the bacteriologic findings on these animals since several workers have found that hemolytic and nonhemolytic streptococci have been isolated from supposedly "normal mice". Therefore, the supposed mutation of pneumococci and nonhemolytic varieties by intraperitoneal injections deserves further investigation. Latent infections due to hemolytic streptococci are quite common in guinea-pigs. They frequently invalidate the diagnostic experiments.

3. **Pneumococcus Infections.**—The extreme susceptibility of the mouse to the pneumococcus is a commonplace of laboratory experience and forms the basis for the rapid isolation of the organism from bacterial mixtures, sputum, etc. Although the mouse may be killed by a smaller dose than the rabbit, it is generally recognized that the rabbit is more susceptible. In an emergency this animal can therefore be used for the typing of pneumococcus strains. Experimentation on guinea-pigs requires careful and critical interpretations since spontaneous pneumococcal infections may be, unusually common in the stock of certain breeders.

4. **Influenza Bacilli.**—It is well to remember that autolyzed influenza bacilli may set up spontaneous streptococcal or pneumococcal infection in guinea-pigs and mice. A general blood infection may be produced regularly when the sputum to be tested contains the symbiotic adjuvants, the cocci.

5. **Tuberculosis.**—The detection of tubercle bacilli by animal inoculation offers several disadvantages. Not infrequently these animals suffer from chronic diseases, such as pseudotuberculosis or Salmonella infections which present gross anatomic lesions readily confused with those of tuberculosis. Spontaneous tuberculous infections of the guinea-pig and rabbit have been reported by many workers and thus may greatly invalidate the significance of the test. In part, these disadvantages may be overcome by a judicious interpretation of the postmortem findings. It is a well-known

fact that, in susceptible animals, the primary localization of the lesion always indicates the avenue of infection. If, for example, after subcutaneous inoculation a tuberculosis of the tracheobronchial lymph nodes with no disease in the inguinal nodes is found, one may conclude that the infection was acquired from extraneous sources.

6. **Plague.**—*B. pseudotuberculosis rodentium* (Pfeiffer) occurs spontaneously in guinea-pigs and may thus interfere with the diagnosis of plague.

7. **Brucella Infections.**—It is not generally appreciated that isolated cases of melitensis and abortus infections have been reported in animals. An extensive epizootic which was caused by a melitensis type and affected 400 guinea-pigs has been reported by Zdrodowski. In view of the wide distribution of the *Brucella* organisms, it is imperative that in the future shipments of guinea-pigs from unknown breeders be scrutinized for *Brucella* infection. Agglutination tests previous to the inoculation of the test material are exceedingly useful.

8. **Anaerobic Infections.**—Those who use guinea-pigs or rabbits for the primary isolation of a pathogenic anaerobe should always remember that certain species may be found as common parasites in the intestinal canal or even in the organs of these animals.

9. **Paratyphoid Infections.**—The fact that *S. aertrycke* orally administered to mice sets up a lethal septicemia, while *B. paratyphosis* B. (Schottmüller) usually does not, is sometimes used as a practical differential test of these 2 organisms. Unfortunately, its value is greatly reduced on account of the widespread occurrence of latent aertrycke infection in rodents. Experimentation with paratyphoid bacilli on mice requires sound judgment. The bacteriologic literature reports such bizarre findings as the transformation of a *B. para-typhosus* into a *S. enteritidis* or *S. aertrycke*.

10. **Virus Infections.**—The controversial literature on experimental encephalitis clearly shows that latent widespread parasitic infections may be responsible for misleading conclusions. Furthermore, the discovery of a new virus by Miller, Andrewes and Swift, in the course of an attempt to reproduce experimental rheumatic fever, furnishes another example of the many obstacles which may continue to render the animal test a diagnostic procedure having considerable complications.

Many more observations could be cited. In particular, attention might be drawn to the deficiency of the hemolytic complement in the blood of certain races of guinea-pigs owing to the absence of the third component (Hyde). Suffice it to emphasize that the bacteriologist who employs the animal as a test object or model in his diagnostic work should be in a position to defend his claims that the lesions or findings are not those of a spontaneous disease, but the result of the experiment. This aim can be met to a great extent if he employs only well-bred animals with a known hereditary history free from bacterial infections and parasitic invasions, maintained and cared for by an experienced personnel, and kept on well-balanced diets in a sanitary and hygienically controlled environment.

## DISEASES OF GUINEA-PIGS

1. **Pneumonia.**—The causative organism in most cases is a hemolytic streptococcus. Occasionally sporadic cases are due to infection with the *pneumococcus*. The *B. bronchisepticus* (*Alcaligenes bronchisepticus*) and Friedländer's bacillus (*Klebsiella*

*pneumoniae*) have been reported as producing pneumonia in these animals. The symptoms are loss of appetite, roughened coat, and rapid breathing.

Autopsy findings are congestion with consolidation of lungs, which may be associated with pleural and pericardial exudates of a serous or fibrinous character.

2. **Abscesses.**—Abscesses result from an infection of the lymph glands with hemolytic streptococci. They often become very large and are usually encapsulated. The contents are a thick and creamy pus from which streptococci can be isolated.

They may become enzootic or even cause epizootics among guinea-pigs. This condition is often called "epizootic lymphadenitis", and the lymph nodes of the neck and axilla are most commonly infected. It is not usually fatal unless the abscesses become large enough to interfere with the function of important organs.

After rupture and drainage the animal usually recovers. It is advisable to incise, remove the pus, and allow good drainage.

3. **Paratyphoid.**—This disease is caused by bacteria belonging to the *Salmonella* group. The organisms usually encountered are closely related to the *S. aertrycke* or the *S. enteritidis*.

The symptoms are loss of appetite, roughened fur, emaciation and weakness. In acute cases death often occurs before symptoms are noted. Outbreaks among laboratory animals may cause considerable loss. The mortality varies from 40 to 70 per cent.

At autopsy, the spleen and liver show the most constant changes. The former is enlarged and soft, studded with small foci or large yellowish nodules and often covered with a plastic exudate. The liver shows small necrotic foci. The intestines are injected and show swollen Peyer's patches. Pleurisy, pneumonia and purulent endometritis may be present.

4. **Pseudotuberculosis.**—This disease is characterized by the formation of whitish nodules in the liver and spleen associated with enlarged lymph glands which often become abscessed. The causative organism is the *B. pseudotuberculosis (rodentium)*, which is a small, coccoid, gram-negative, nonmotile and distinctly bipolar organism. It will grow on ordinary culture media under aerobic conditions. It is difficult to demonstrate in chronic lesions. It can be definitely identified by an agglutination test with specific serum.

Three clinical types are recognized: (a) Septicemic type (death in 1 or 2 days); (b) emaciation and diarrhea (death in 3 to 4 weeks), and (c) glandular type.

The diagnosis is made by bacteriological examination and the characteristic lesions.

Control measures are not recommended because of lack of knowledge concerning the epidemiology of the disease.

5. **Tuberculosis.**—Natural infections of guinea-pigs with tubercle bacilli are not common. Normal guinea-pigs can readily contract the disease from tuberculous cage mates or even from animals in the same room.

## DISEASES OF RABBITS

1. **Nasal Catarrh (Snuffles).**—This condition may be caused by bacterial infection or by coccidia. When due to bacteria it is spoken of as "snuffles" and when due to coccidia (*Eimeria stiedae*) as "nasal coccidiosis".

The organisms most commonly found are *B. lepi-septicus* (*Pasteurella cuniculicida*)

and *B. bronchisepticus* (*Alcaligenes bronchisepticus*). The latter, when present, is usually associated with the former.

The diagnosis of nasal coccidiosis is made by finding the coccidia in the nasal secretions.

The symptoms are sneezing, nasal discharge which is recognized by wetting of the hair around the nasal openings, or the presence of mucus and emaciation. Inflammation of the eyes is often present in cases due to coccidia. In the bacterial type the infection may extend to the lungs and cause pneumonia.

2. **Pneumonia.**—This is usually the result of extension of infection from the upper respiratory tract. The organisms usually found are the *B. leipsepticus* and the *B. bronchisepticus* (*Alcaligenes bronchisepticus*).

3. **Coccidiosis.**—This disease is caused by coccidia (*Eimeria stiedae* and *Eimeria perforans*), which are among the commonest parasites found in the rabbit. Although they are found in the intestinal contents of apparently normal rabbits, they are capable of invading the epithelium and causing a mild catarrh to a severe type of enteritis (intestinal coccidiosis).

They commonly invade the epithelium of the bile ducts, gallbladder and liver. Multiple small white nodules result from their invasion of the liver (hepatic coccidiosis).

The nasal form of the disease has been given above under nasal catarrh of rabbits (snuffles).

The diagnosis is made by finding the coccidia in association with enteritis or nodules. The organism is readily demonstrated by placing the contents of a liver nodule, bile, or scrapings of intestinal mucosa on a slide and examining the unstained specimen.

The oöcysts appear as oval bodies from 15 to 25 micra in length.

There is no treatment. Preventive measures should consist of frequent cleaning of cages, separating animals, avoiding grouping, raising floor with wire mesh so droppings can pass through, etc.

4. **Ear Mange.**—This is caused by a mite *Dermatocoptes* (*Psoroptes*) *cuniculi*. The disease is characterized by the formation of thick crusts or deposits on the inside of the ear.

The diagnosis is made by examining scrapings from the ear, unstained, under low power, for the mite.

Infected animals should be separated and treated locally. Remove the scabs and apply one of the following:

Mercuric chloride .....	1 part
Glycerin .....	100 parts
Ethyl alcohol (50 per cent) .....	200 parts

or

Oil of caraway .....	1 part
Almond oil .....	10 parts
Ethyl alcohol (90 per cent) .....	3 parts

5. **Giardiasis.**—*Giardia* may be found quite commonly in the small intestines of rabbits. Whether or not they are harmful is questionable.

6. **Parasitic Cysts.**—Cysts of the dog tapeworm (*Taenia pisiformis*) are commonly found in the mesentery of the rabbit. They are spoken of as “bladder worms” and often occur in large numbers and occasionally in the liver.

The general health of the animal is not affected.

### DISEASE OF MICE

**Mouse Typhoid (Paratyphoid).**—This is the commonest infection and is due to bacilli of the Salmonella group, especially *S. aertrycke* and *S. enteritidis*. Spontaneous epizootics occur, causing considerable loss; the mortality is high, ranging from 34 to 95 per cent.

Susceptibility varies in different breeds and strains of mice and is influenced by diet and environment.

The diagnosis is established by isolating an organism belonging to the Salmonella group from the spleen or other organs.

### DISEASES OF RATS

1. **Lung Disease.**—This is a respiratory infection prevalent among albino rats. Young rats are not susceptible. The symptoms are loss of appetite, rhinitis, conjunctivitis and labored breathing; as the disease becomes chronic there is much depression. The mortality is high.

At autopsy the lungs are always affected; unresolved pneumonias, catarrhal bronchitis with bronchiectases, abscesses and pleurisy are common findings.

Various organisms of dissimilar groups have been isolated from these lesions, none of which have been recognized as the cause. The etiology therefore is still undetermined.

2. **Paratyphoid.**—This disease is due to members of the Salmonella group, particularly the *S. enteritidis* type. The symptoms in acute cases are diarrhea and bloody crusts around the eyes.

The lesions found at autopsy are swollen spleen, multiple necrotic foci, enlarged lymph glands and Peyer's patches. Chronic cases may fail to show any lesions. The organisms can be readily isolated from the spleen and liver.

### GENERAL DIRECTIONS FOR THE INJECTION OF ANIMALS

1. Select an appropriate size syringe that does not leak upon being tested with water. Nothing is more unsatisfactory than a leaking syringe for not only may the hand become soiled, but an unknown quantity of inoculum is lost.

2. Remove the plunger from the barrel and sterilize all the parts by boiling for at least several minutes. An all-glass syringe or a glass barrel and metal plunger are the most satisfactory. The old-fashioned syringe with washers and rubber-tipped plunger should find no place in a laboratory.

3. After cooling, expel the water and load the syringe. This may be done by drawing the fluid directly into the syringe and measuring the dose by its markings or by pipeting the exact dose into a sterile Petri dish or capsule and drawing up in the syringe.

4. The animal should be fastened or held firmly and in an easy position. Everything should be in readiness so that the injections may be given thoroughly and carefully.

5. In preparing the inoculum, care should be exercised that no solid particles enter the syringe. Aside from possibly blocking the needle and interfering with the injection, the subcutaneous injection of small fragments may do no particular harm, but in intravenous inoculation they may cause fatal embolism.

6. Air bubbles should be removed. The injection of small bubbles of air into subcutaneous tissues may cause no harm, but when injected into veins they may cause serious disturbances or immediate death. To avoid this, the syringe, after being filled, should be held vertically, with the needle uppermost. The needle should be wrapped in cotton soaked in alcohol and the piston of the syringe pressed upward until all the air is expelled from the barrel and the needle. If a drop of inoculum is forced out, it will be collected on the cotton, which should be burned immediately or placed in a disinfectant.

7. Injections should be given slowly.

8. When it is necessary to incise the skin in order to reach a vein, an anesthetic may be given. With superficial veins, and in subcutaneous inoculations, the injections may be given so readily and easily that no more pain can be felt than that which accompanies similar injections in human beings.

9. When it is necessary to remove the hair from the area to be injected, a small area may be clipped as closely as possible, followed, if necessary, by shaving. In guinea-pigs the hair may be plucked out; or clip the hair and apply a paste of equal parts of barium sulphide and cornstarch mixed with water. Leave this on for 3 or 4 minutes, wash thoroughly with warm water and dry with a towel. This is a particularly good method when large areas of skin are to be prepared. As it may cause irritation, it is well to remove the hair a day in advance of injection.

10. Before injection cleanse the skin with 70 per cent alcohol or wipe with 1:1000 mercuraphen or other disinfectant.

### **TECHNIC OF INTRACUTANEOUS INJECTION**

1. Select white animals or white areas if skin reactions are to be elicited such as allergic reactions, diphtheria toxin reactions, etc.

2. Use a 1 cc. syringe with No. 26 needle.

3. Prepare the skin. Pinch up a fold and insert the needle (lumen up) as superficially as possible. A raised, white, anemic spot showing the pits of hair follicles indicates a successful injection. Owing to thin skins of rabbits and guinea-pigs, the injection is by no means easy or simple and requires practice. The amount injected should not exceed 0.1 cc.

### **TECHNIC OF SUBCUTANEOUS INJECTION**

1. Injections are usually given in the median line of the abdominal wall or in the groin.

2. Have the animal held firmly by an assistant or firmly secured to the animal operating table.

3. Clip the hair where injection is to be made—it is not always necessary to shave the area. Apply a 2 per cent iodine in alcohol solution.

4. Pinch up a fold of skin between the forefinger and the thumb of the left hand; hold the syringe in the right hand, insert the needle into the ridge of skin between the finger and thumb, and push it steadily onward until the needle has been inserted about an inch. Care must be exercised not to enter the peritoneal cavity. Relax the grasp of the left hand and slowly inject the fluid. If the skin is raised, this shows that the injection is subcutaneous. If it is not, the needle should be slightly withdrawn and inserted.

5. Withdraw the needle and, at the same time, cover the puncture with a wad of cotton wet with alcohol. A touch of flexible collodion over the puncture completes the operation.

6. If a *solid* inoculum is to be injected, raise a small fold of skin with a pair of forceps and make a tiny incision through the skin with a pair of sharp-pointed scissors.

7. With a probe, separate the skin from the underlying muscles to form a funnel-shaped pocket.

8. By means of fine-pointed forceps or a glass tube syringe, introduce the inoculum into this pocket and deposit it as far as possible from the point of entrance of the instrument.

9. Close the wound with collodion and cotton. A single stitch with fine thread may be necessary.

### TECHNIC OF INTRAMUSCULAR INJECTION

1. These injections are usually made into the posterior muscles of the thigh or into the lateral thoracic or abdominal muscles.

2. Clip away the hair over the selected area, cleanse, etc., as for subcutaneous injection.

3. Steady the skin over the selected muscles with the slightly separated left forefinger and thumb.

4. Thrust the needle of the syringe quickly into the muscular tissue and slowly inject the fluid.

### TECHNIC OF INTRAVENOUS INJECTION

**Rabbit.**—1. The posterior auricular vein along the outer margin of the ear is better adapted than a median vein for this purpose.

2. If a number of injections are to be made, commence as near the tip of the ear as possible as the vein may become occluded with thrombi and subsequent inoculations may then be given nearer and nearer the root of the ear.

3. The animal should be held firmly as the slightest movement may result in piercing the vein through and through and require reinsertion of the needle. This is accomplished satisfactorily by placing the rabbit upon the edge of the table and holding it there firmly by grasping the neck and front quarters, the assistant at the same time compressing the root of the ear with the thumb and forefinger.

4. If the hair is long, clip it.



5. The ear is struck gently with the fingers and washed with alcohol and xylol; the friction will render the vein more conspicuous.

6. The ear is grasped at its tip and stretched toward the operator, or the vein may be steadied by rolling the ear gently over the left index finger and holding it between the finger and thumb.

7. The inoculum should be free from solid particles and all the air excluded from the syringe. As a general rule, the amount injected should be as small as possible and the temperature of the inoculum be near that of the body. If the syringe is filled shortly after sterilization, when it has cooled enough to be comfortably hot to the touch, the heat will warm the injection fluid and not be hot enough to cause coagulation.

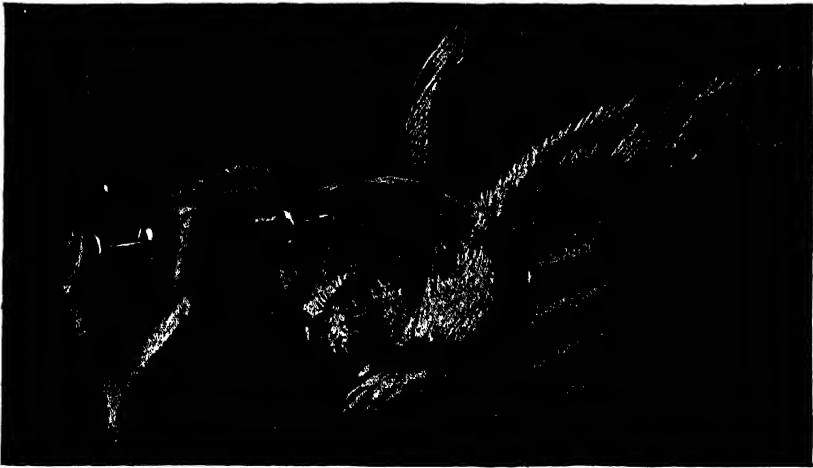


FIG. 10.—INTRAVENOUS INJECTION OF A RABBIT

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

8. Hold the syringe as one would hold a pen, and thrust the point of the needle through the skin and the wall of the vein until it enters the lumen of the vein (Fig. 10).

The wooden box shown in Figure 11 is very convenient for holding rabbits for intravenous injection or for bleeding from the ears.

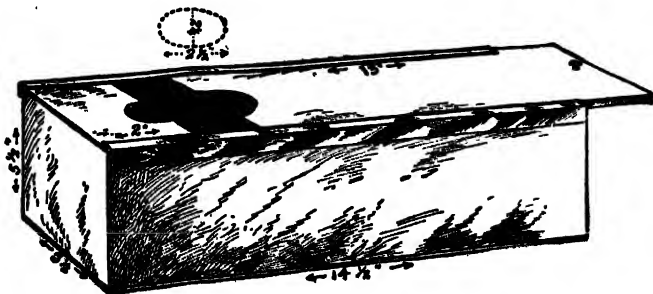


FIG. 11.—A WOODEN BOX FOR RABBITS

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

9. Direct the assistant to release the pressure at the root of the ear and *slowly* inject the inoculum. If the fluid is being forced into the subcutaneous tissue, which will be evident at once by the swelling which occurs, the injection must cease and another attempt made.

10. The needle is quickly withdrawn, a small piece of cotton moistened with alcohol placed upon the puncture wound, and firm compression applied.

Wash the ear thoroughly with alcohol and water to remove xylol, otherwise a lowgrade inflammation will follow which will render subsequent injections more difficult.

**Guinea-Pig.**—1. The large superficial vein lying on the dorsal and inner aspect of the hind leg of the guinea-pig is well adapted for intravenous injection. Occasionally, however, the vessel may run anteriorly. To use the above described vessel for intravenous administration, a special operating board is required. The board is similar to an ordinary animal board except that the end to which the hind legs of the animal are tied has a U-shaped piece cut from it. The board is mounted near the center on an extension shaft which is fitted with 2 joints, the one at the end to which the board is attached being a ball-and-socket joint and the other an adjustable swivel joint. The shaft is screwed into a metal base which has sufficient weight to hold the board steady when placed in any position. A single operating board, however, may be used, as shown in Figure 12.

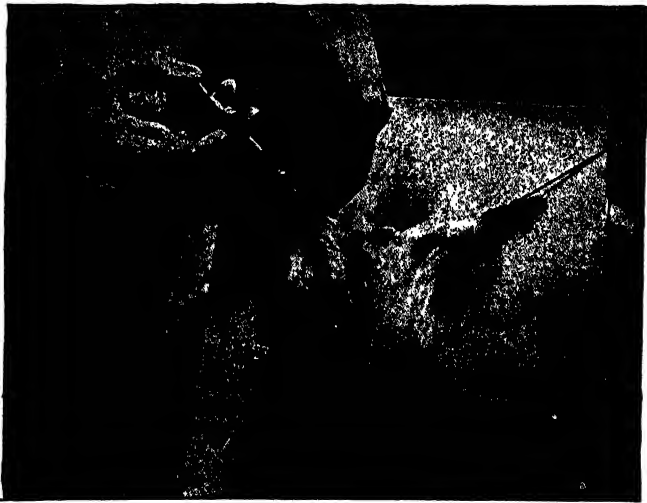


FIG. 12.—ROTH'S METHOD FOR INTRAVENOUS INJECTION OF THE GUINEA-PIG  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

2. The procedure for making the injection is as follows: With the board properly placed in a horizontal position, the animal is tied to it securely, abdomen downward, by means of strings. The board is then placed in a vertical position and rotated on its vertical axis slightly so as to bring the dorsal aspect of the right hind leg into view. After clipping the hair from the leg and shaving it, the leg is lifted up slightly by the first or first and second fingers, and the vein dilated by suitable compression. The vessel can now usually be seen through the skin. A small incision, usually about

$\frac{1}{4}$  inch long, is made across the leg from the outer lower to the upper and inner aspect, but a trifle to the left of the vessel. The subcutaneous tissue is then pushed aside with fine-pointed forceps, thereby permitting the vessel to come into view.

3. The vessel is then entered directly or in the same manner as has been described for the rat—that is, by passing the needle of the tuberculin syringe through the fascia and muscles to the left of the vessel and then entering the vessel from the side. The vessel, when dilated, permits the ready entrance of a No. 23 gauge needle. However, the needle usually employed is a No. 26 gauge,  $\frac{5}{8}$  inch in length. The needle is always introduced well into the lumen of the vein. If entrance into the vessel is direct, subsequent hemorrhage may be controlled quite readily by pinching it with a small forceps.

4. Injections may be given also in one of the external jugular veins as follows:

(a) A small roll is placed under the neck of the animal to render the operative area tense and more easily accessible.

(b) A few drops of ether may be given by an assistant although one soon learns to expose the vein quickly and there is practically no pain after the skin has been incised.

(c) Assistant is directed to hold the head backward in the median line.

(d) Pick up the skin just above and in the middle of the space between the shoulder and the tip of the upper end of the sternum — just above and about in the center of the area where a clavicle in the human would be situated. With small sharp scissors incise the skin for about  $\frac{1}{2}$  inch. Separate the subcutaneous tissue gently with forceps; a large vein at once comes into view. Gently dissect it free for about  $\frac{1}{4}$  inch.

(e) Pick up the vein with a pair of fine forceps, insert the needle of the syringe gently in the long axis of the vein and slowly inject the fluid.

(f) Withdraw the needle and apply firm pressure with a wad of clean gauze or cotton. It is not necessary to tie off the vein. A stitch may be inserted to close the skin wound and flexible collodion applied.

**White Mouse.**—1. It has been found that the lateral veins of the tail of the white mouse are best suited for intravenous administration purposes. The tail must be free from localized or generalized thickening of the epidermis so as to permit the ready entrance of a No. 23 gauge needle. The use of a rather long needle, 1 inch in length, is essential; it does not bend easily and therefore can be directed forward more readily than a smaller one.

2. A mouse weighing between 15 and 20 grams practically always possesses a soft, pliable tail which can be used without any preparation. When a mouse weighing over 20 grams is used, the lateral veins of the tail are usually covered with rather dense tissue, which precludes their use unless the tail is immersed for about a half minute in warm water (about 50° C.). This procedure both softens the skin and dilates the underlying vessels so that the latter may be successfully used.

3. For holding the mouse, a small tin mailing tube attached to an iron stand is employed (Fig. 13). One end of the metal mailing tube is fitted with a cork having at the circumference a V-shaped opening which will admit the tail. The other end of the tube contains several small openings to admit air.

4. The mouse is grasped by the tail with the thumb and forefinger of the left hand and placed in the above-described metal mailing tube, and the cork is inserted so that the tail protrudes through the V-shaped opening. The tail is then straightened by

gentle but firm traction and without twisting. The dorsal vein should then appear above, and each lateral to the left and right, respectively.

5. The syringe, usually a 1 cc. all-glass, tuberculin type, graduated to 0.01 cc., is balanced between the first and middle fingers of the right hand, the hand resting on the little finger; the thumb is thus free to operate the piston of the syringe. With the syringe held nearly parallel to the tail, the needle is pushed through the skin over one of the lateral veins (usually the left) and then anteriorly and downward into the vein. If an entrance into the vessel is not effected, either raising or lowering the point of the needle while advancing it further will usually succeed in locating the lumen of the vessel.

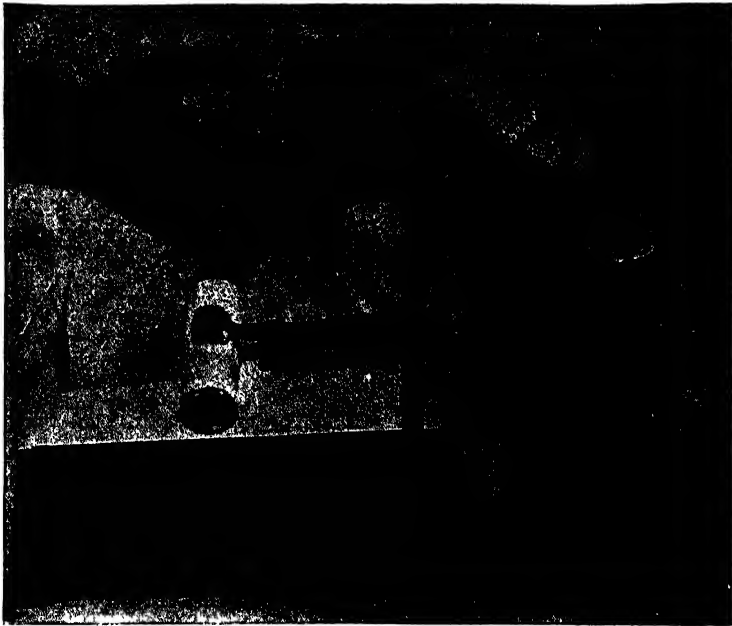


FIG. 13.—ROTH'S METHOD FOR INTRAVENOUS INJECTION OF THE MOUSE  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

**White Rat.**—1. The animal is tied securely by the legs, back downward, to a flat operating board by means of strings long enough to permit the hind legs to be lifted easily.

2. At the end of the board to which the head is tied are 2 glass pegs about 1 inch long set in at an angle in order to hold the string which is looped over the front legs of the animal. Nails in the other end of the board receive the strings which are looped to the hind legs (Fig. 14).

3. After shaving the hair over the skin area covering the saphenous vein, the left foot is grasped between the third and middle fingers of the left hand and an incision about  $\frac{1}{4}$  to  $\frac{1}{2}$  of an inch long is made about  $\frac{1}{4}$  of an inch to the left of and parallel to the vein. The skin is then rolled over to the right with the first finger of the left hand by drawing the skin on the back of the leg to the left. This will bring the vessel into view. An assistant then makes compression to dilate the vessel. If a syringe is used, it

is preferable to employ a 1 cc. all-glass tuberculin type, graduated to 0.01 cc. and fitted with a No. 26 gauge needle,  $\frac{5}{8}$  inch in length. The syringe is balanced between the first and middle fingers of the right hand, the hand resting on the little finger; the thumb is thus free to operate the piston of the syringe. The needle is then passed through the fascia and upper surfaces of the muscles, about  $\frac{1}{8}$  inch to the left of the vein and almost parallel to it. Advancing the needle slightly farther, the direction is changed so that the needle will enter the vein from the side. After the injection is made, the skin which was pulled to the right to permit the vessel to come into view, is released and this skin flap and the muscles act as effective mechanical checks to hemorrhage, which is quite profuse, if the needle is inserted directly into the vein.

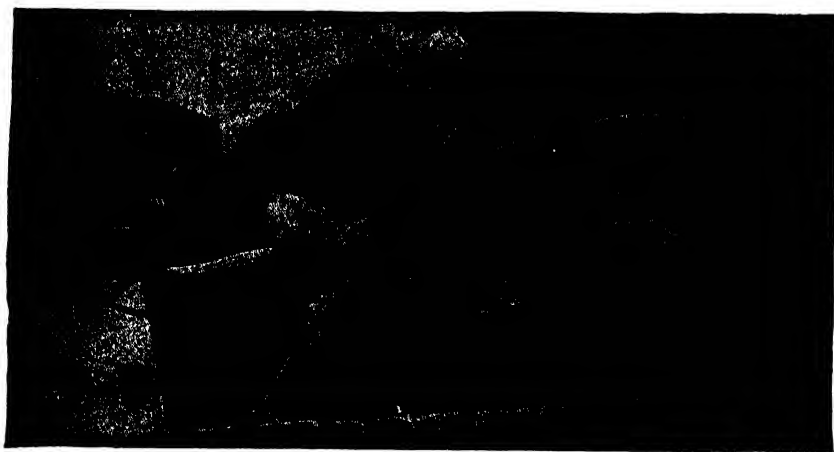


FIG. 14.—INTRAVENOUS INJECTION OF THE WHITE RAT EMPLOYING THE FEMORAL VEIN  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

4. If a buret is employed and the injection made by gravity instead of by means of a syringe, a flexible rubber tube is attached to the buret, while the other end of the tube carries a glass tube which is drawn out and ground to fit a No. 25 gauge needle 1 inch long. The glass tube is handled in the same way as the syringe, and the vessel is entered in the same manner as described under the syringe method.

5. An *external jugular vein* may be used instead and has the advantage of being larger.

The animal is tied to the operating board in the same manner and a wad of cotton placed under the shoulder. With a piece of cotton an assistant holds the head backwards and to one side, rendering the tissues of the operative area tense and firm (Fig. 15).

The skin is touched with alcohol and a small incision made just above and about in the center of the area where a clavicle in the human would be situated. The subcutaneous tissues are gently dissected and the vein exposed, which becomes very prominent with respiratory movements. It is well not to attempt entering the vein until it is thoroughly exposed, as otherwise one may infiltrate the tissues over and about the vein by failure to enter it properly. As soon as the injection has been given the animal is quickly released and the wound requires no attention as infection practically never occurs and healing is rapid.



FIG. 15.—INTRAVENOUS INJECTION OF RAT EMPLOYING THE EXTERNAL JUGULAR VEIN  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

**Dog.**—1. Dogs may be injected through the external jugular or popliteal veins. The animal should be fastened to an operating table.

2. There is a small vein just beneath the skin in the median line, along the anterior surface of the leg, which is readily accessible. Clip away the hair and disinfect with iodine and alcohol. Direct the assistant to grasp the thigh just above the knee to distend the vein and prevent movement, and make a small incision directly in the median line. A small vein is seen at once. Dissect free or pick up gently with fine forceps and insert a small sharp needle. The injection can thus be readily given. Withdraw the needle, apply firm pressure, and insert a single stitch. Bind the wound with a few turns of a gauze bandage or seal with collodion and cotton.

### TECHNIC OF INTRACARDIAL INJECTION

1. Guinea-pigs may be injected by the intracardial route instead of intravenously. The technic is not, as a rule, more difficult, and no ill effects are noticed. Not infrequently, however, attempts to inject in the heart fail and frequent trials are not permissible on account of the danger of injuring the organ.

2. The animal is tied to an operating board or held firmly by an assistant; an anesthetic may be given.

3. Determine the point of maximum pulsation to the left of the sternum by palpation, and quickly insert a thin, sharp needle at the selected area. A flow of blood indicates that the needle has entered the heart. Attach the previously filled syringe and slowly inject the contents.

4. Detach the syringe in order to make sure that the injection was intracardial as intended, which is indicated by a flow of blood; then quickly withdraw the needle. The puncture wound may be sealed with collodion.

**TECHNIC OF INTRAPERITONEAL INJECTION**

**Rabbit.**—1. Clip the hair and shave an area about 2 inches in diameter in the median abdominal line just below the umbilicus. Apply 2 per cent iodine in alcohol.

2. Direct an assistant to hold the animal firmly, head down. With the animal in this position the loops of intestine tend to sink toward the diaphragm, leaving an area above the bladder which is sometimes free of intestines (Fig. 16).



**FIG. 16.—INTRAPERITONEAL INJECTION OF RABBIT**

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

3. The syringe is grasped firmly and the needle inserted beneath the skin for a short distance in the direction of the head in the long axis of the animal, when the hand is raised and the needle forced forward through the peritoneum. When the peritoneum has been entered this is evidenced by a relaxation of the abdominal muscles. The needle is then withdrawn slightly and the injection made.

**Guinea-Pig.**—1. Direct an assistant to hold the animal firmly upon its back. This is better than fastening it to an operating table for it permits relaxation of the abdominal wall when the injection is to be made.

2. Pluck the hair in the median abdominal line. A small area may be shaved, although this is not necessary. Disinfect with an application of iodine in alcohol.

3. With the left forefinger and thumb pinch up the entire thickness of the abdominal parietes in a triangular fold and slip the peritoneal surfaces over each other to ascertain that no coils of intestine are included.

4. Grasp the syringe in the right hand and insert the needle into the fold near its base.

5. Release the fold and inject the fluid. If a swelling forms, this shows that the needle is in the subcutaneous tissues and another attempt should be made to enter the peritoneum.

6. It may be difficult to pinch up the parietes without including the intestine. In such case straighten out the animal and stretch the skin between the left forefinger and thumb. Insert the needle obliquely until it is beneath the skin. A slight thrust suffices to pierce the peritoneum, when the abdominal muscles will be felt to relax. Withdraw the needle slightly and inject the fluid.

7. Seal the wound with a touch of collodion.

**Mouse.**—The technic is practically the same (Fig. 17).

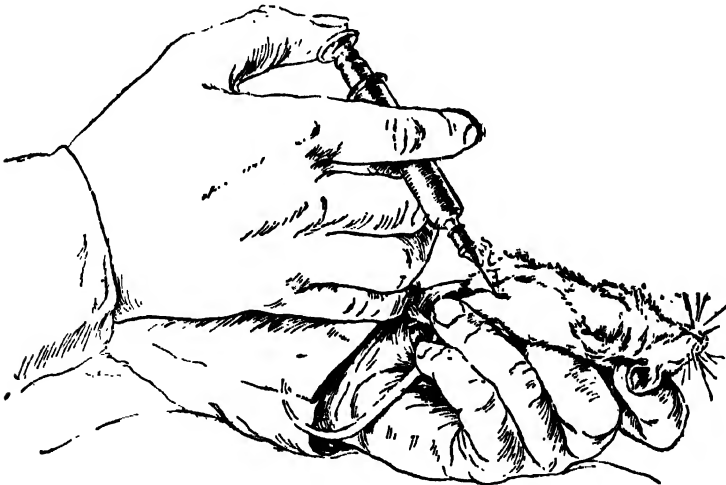


FIG. 17.—INTRAPERITONEAL INJECTION OF MOUSE

(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)



### TECHNIC OF SUBDURAL INJECTION

**Rabbit.**—1. Use a No. 18 gauge needle which has been cut off and sharpened to about 3/16 of an inch in length, and a 1 cc. syringe. Sterilize by boiling and fill with inoculum.

2. Shave or clip the hair over the site of injection, which is located a few centimeters posterior to and on a line with the outer canthus of the eye. In this region a small horizontal groove can be detected by feeling with the finger nail. The bone at this point is thin.

3. Place the needle in the groove and force it through the bone into the cranial cavity.

4. Inject the material slowly.

### TECHNIC OF TESTICULAR INJECTION

**Rabbit.**—1. Sponge the scrotum with tincture of iodine.

2. Fill sterile syringe with material to be inoculated. Use a No. 22 gauge needle about 5/8 of an inch in length.

3. Insert the needle through the skin of the scrotum into the substance of the testicle forced into the scrotum by gentle massage.

4. Inject the material.

### METHODS FOR OBTAINING BLOOD FROM ANIMALS

**Rabbit.**—*From the Ear Veins.*—1. Flip an ear vigorously with the hand, and rub with xylol and alcohol. The xylol produces marked congestion and afterwards should be carefully removed with alcohol and water as it produces a lowgrade inflammatory reaction.

2. Puncture a marginal vein with a large needle. The blood will flow quickly in drops, and practically any amount up to 2 or 3 cc. or even more may be collected in a centrifuge or test tube (Fig. 18). For making preliminary tests of serum during immunization, 2 cc. of blood are usually sufficient. Bleeding may be checked by making firm pressure over the puncture.

Another good method is to place the animal in the box shown in Figure 11, turning it up on end and so that the animal's head is down. This permits one person to bleed the animal without assistance.

*From the heart.*—1. Tie the animal securely to a board and clip the hair from an area of the chest about 1½ inches in diameter.

2. Determine the point of maximum pulsation and disinfect with tincture of iodine.

3. If 5 to 20 cc. of blood are required, use a sterile syringe fitted with a No. 17 to 19 gauge needle. If more than 20 cc. are required or the animal is to be exsanguinated, use a 200 cc. bottle with a rubber stopper fitted with 2 pieces of glass tubing, one connected with rubber tubing for suction and the other connected by means of heavy-walled rubber tubing with the needle. Sterilize in an autoclave.

4. If the worker is experienced, ether may be omitted as it may cause more discomfort than the puncture; otherwise etherize lightly.

5. Enter the needle at the point of maximum pulsation, applying gentle suction.
6. Approximately 20 cc. of blood can be taken from a 2000 gram rabbit at intervals of 2 or 3 weeks.

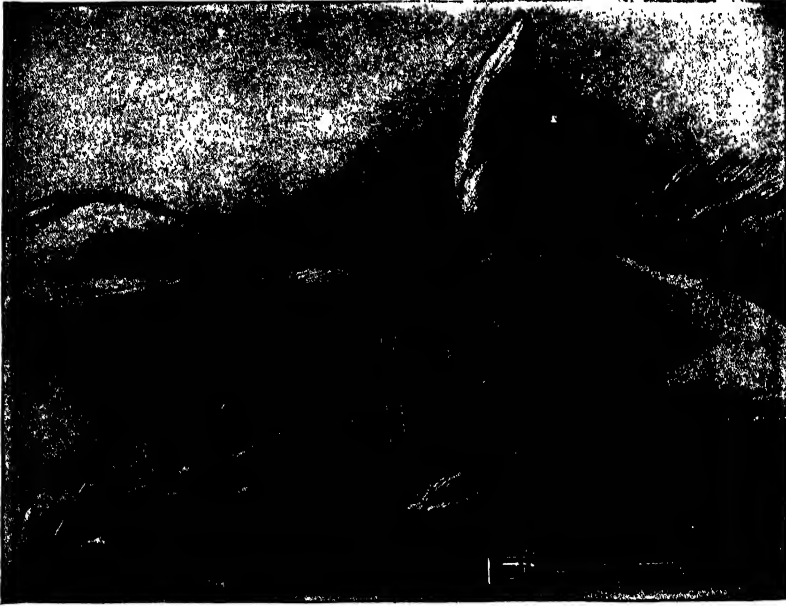


FIG. 18.—METHOD OF BLEEDING A RABBIT FROM THE EAR

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

*From the Neck.*—1. Clip the hair from the neck and disinfect with 70 per cent alcohol or 1 per cent lysol solution; dry with a towel.

2. While an assistant holds the animal head down, rapidly sever the neck on both sides with a razor or sharp scissors (avoiding the trachea and esophagus), and collect the blood by a funnel into centrifuge tubes or a dish. Or, the following may be used: With a sterile knife cut the skin longitudinally and expose the neck muscles for a considerable distance.

3. The assistant then holds the animal upright over a sterile dish or a large sterile funnel, emptying into a cylinder or 50 cc. centrifuge tube.

4. The operator stretches the neck by carrying the head backward, and severs the large vessels on one or both sides of the neck with a sharp sterile scalpel or razor, avoiding opening the trachea and esophagus.

5. After bleeding, the dish is covered or the tube plugged and set aside for the serum to separate.

**Guinea-Pig.**—The animal is anesthetized with ether or stunned with a sharp blow and the large vessels of the neck on one side are exposed by a longitudinal incision. These are severed and the blood is collected in a Petri dish or in a centrifuge tube by means of a funnel. Or, by means of a sharp-pointed scissors, the vessels on one or both sides of the neck may be incised transversely at one cut, inserting the blade deeply and close to, but avoiding, the trachea and esophagus.

Blood may be obtained by aspiration from the heart of the living animal. A syringe fitted with a No. 20 or 22 needle is employed. The animal is fastened to a board or held by an assistant. It is then lightly anesthetized. The point of maximum pulsation is determined and the needle slowly entered into the right chambers of the heart.

As a general rule, 5 to 15 cc. of blood may be obtained by gentle suction, the amount depending upon the size of the animal. Large male animals (700 grams or more) are recommended and may be used every 2 to 3 weeks. After withdrawal of the needle the animal rapidly recovers, although occasional bleeding may follow into the pericardial sac (Fig. 19).



FIG. 19.—METHOD OF SECURING BLOOD FROM THE HEART OF A GUINEA-PIG

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

**Sheep.**—Blood may be obtained easily from a freshly killed animal. The first flow of blood is discarded and a portion of the remainder is collected in a large, sterile, thick-walled flask containing glass beads. By shaking vigorously the blood is defibrinated if one desires to obtain corpuscles, or the blood may be collected in a cylinder and defibrinated by whipping with glass rods.

In large laboratories it is usual, however, to keep a sheep and to remove the blood as it may be required. Small amounts may be obtained from the ear vein, larger quantities from an external jugular vein in the following manner:

1. One person may do the bleeding alone although the aid of an assistant is usually necessary. This is especially so if the animal is large, powerful and inclined to be vicious.
2. The sheep is thrown on its back and the head is held on the knees of an assistant seated on a low box or stool.

3. The operator may straddle the animal to hold down the forefeet, although this is not necessary unless the animal is vicious.

4. The wool on the left side of the neck is clipped closely with scissors and alcohol applied.

5. The operator then grasps the neck low down with the left hand, and by means of the thumb exerts pressure over the base of the neck. The external jugular vein will be found in a groove between the omohyoid and sternomastoid muscles. Firm pressure over the base of the neck usually distends the vein, which may be seen or easily felt. After locating the vein the pressure should be released for an instant, when the distention will disappear. In this way the operator may be more certain that he has located the vein.

6. A sterile stout needle, at least 2 inches in length and provided with a trocar and special shank for firm grasping, is passed quickly into the distended vein in an upward and inward direction. It is essential that the needle be sharp, otherwise it will be turned aside by the wall of the vein. The end of the needle must not have too long a bevel, or the point will pierce the opposite wall before the body of the needle is well within the vein. The trocar is now removed and blood collected in a flask or bottle and defibrinated with glass beads or rods. A short piece of rubber tubing may be attached to the needle. A suction apparatus is not needed because the flow of blood is good so long as pressure is preserved over the vein at the base of the neck.

7. When the required amount of blood has been secured, pressure is released and the needle quickly withdrawn. Bleeding ceases at once and the neck is then washed with alcohol.

8. By this method the same vein may be used over and over again for several years. We have never known infection to occur although the gradual formation of scar tissue about the site of the puncture may interfere with the operation.

**Horse and Cow.**—1. Clip the hair on the side of the neck over the jugular vein.

2. Apply alcohol.

3. Apply pressure with thumb until the vein becomes prominent.

4. Insert a No. 16 to 18 gauge needle first through the skin and then into the vein.

5. After collecting blood, remove needle and wipe with alcohol.

**Hog.**—1. Cleanse the tip of the tail and wipe with alcohol.

2. Lay the end of the tail on a block of wood and chop off about  $\frac{1}{4}$  to  $\frac{1}{2}$  inch with razor.

3. Allow the blood to run into a sterile container.

4. When required amount is obtained, tie a string around the tip of the tail to prevent further bleeding.

**Dog.**—*From Jugular Vein.* 1. Muzzle and lay on side.

2. Clip hair and then shave the side of the neck over the jugular vein.

3. Have the head extended and apply pressure over jugular vein until it becomes prominent. If the tissues around the vein are loose it is well to draw the tissues down at the same time the pressure is applied, thus rendering the vein immovable.

4. Insert the needle through the skin and then into the vein.

*From the Radial Vein.*—1. Muzzle and lay on table in natural position and hold one front foot extended forward.

2. Apply firm pressure around the proximal end of the radius, or humeral radial articulation. The radial vein will become prominent along the anterior surface, from which blood can be drawn with a syringe and needle.

*From the Saphenous Vein.*—1. Muzzle and place on side.

2. Apply pressure on the inner thigh region or around the thigh or femoral region. The saphenous vein will become prominent and blood can be removed at a point where it passes very superficially over the outer tibial region.

**Fowl.**—*From the Comb.*—Clip off a small piece of one of the points of the comb. Sufficient bleeding will occur to furnish blood for blood counts, hemoglobin estimations, smears, etc.

*From the Humeral Vein.*—1. Hold the bird on its side so that the breast is presented toward the operator. Turn the top wing back.

2. Insert the needle (No. 21 gauge) into the humeral vein which is situated in the loose fascia on the inner side of the wing in the humeral region.

3. Withdraw the blood and place in tube as quickly as possible as the blood of fowls coagulates rapidly.

### TECHNIC OF POSTMORTEM EXAMINATIONS

1. Autopsies should be performed as soon after death as possible, especially if bacteriological examinations are required.

2. Select suitable instruments and have a separate set for each step if bacteriological examinations are to be made (in which case boil all instruments for at least 5 minutes).

3. Fasten the animal on a board or tray exposing the ventral surface. Disinfect the hair with 1 per cent cresol and examine the skin at the point of inoculation for ulcerations, etc.

4. Incise the skin from the neck to the pubis. Cut the skin at right angles at the ends of the cut.

5. Separate the skin and lay the flaps back on each side, exposing the entire abdomen and thorax. Note the condition of the subcutaneous tissue, axillary and inguinal lymph glands.

6. Make cultures of the peritoneal fluid at this time by puncturing the peritoneal wall with point of capillary pipet or needle attached to a syringe.

7. With fresh instruments, cut through the peritoneal wall from diaphragm to the pubis. Make right angle cuts to form flaps which can be laid back to expose the organs.

8. Examine and make necessary cultures of the abdominal organs.

9. With blunt-pointed scissors cut through the costal cartilages, making a V-shaped incision. Lay back the flap and expose the thoracic organs.

10. If a blood culture is to be made, lift the heart and hold it in position with a hemostatic forceps. Cut the pericardium and sear an area of the right ventricle with a hot instrument; make a short incision with a sterile scalpel, withdraw blood with a sterile pipet or a sterile loop to a suitable culture medium and make smears on slides.

11. If histological examinations are desired, remove small pieces of tissue and place them in a Zenker's solution or 4 per cent formalin; use 5 to 6 times as much solution as tissue.

12. Dispose of the animal by burning or place it in a 1 per cent solution of crude cresol for disinfection.

13. Those who are not familiar with the anatomy of the various experimental animals should consult texts on this subject 4-7.

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# METHODS FOR THE PREVENTION AND TREATMENT OF LABORATORY ACCIDENTS

The modern laboratory worker is surrounded by many dangers, especially from infection in the conduct of necropsies and the handling of infectious material in bacteriological examinations, as well as from scalds, burns, the accidental swallowing of corrosive poisons, the inhalation of poisonous fumes, breaking of glassware, etc. This is especially true of inexperienced workers who are likely to be ignorant of potential dangers or may engage in conversation and other distractions while handling and measuring infectious material, cultures, acids, alkalis, etc.

## PREVENTION OF ACCIDENTS

1. Good rubber gloves should be worn always in the conduct of necropsies of animals or individuals, and the handling of fresh infectious tissues (anthrax, syphilis, tuberculosis, etc.); and great care should be taken against accidental pricking of the fingers with sharp edges of bone and needles as well as against cuts with knives, saws, etc.

2. Pipets employed for transferring or measuring cultures of virulent bacteria like tubercle, diphtheria, and typhoid bacilli, *Br. abortus*, etc., should have the mouth ends plugged with cotton or a piece of rubber tubing with a glass mouthpiece should be attached for filling and expelling. Various syringes are available also. Likewise in pipeting acids, alkalis and other poisonous solutions, the worker should use these precautions and exercise great care. It is particularly important to keep one's mind on the work and not to engage in conversation or other distractions.

3. The hands should be kept free of cuts and abrasions, particularly around the fingernails, and carefully washed with soap and water followed by immersion in a disinfecting solution after handling infectious material and before meals.

4. Table tops should be wiped carefully with a disinfectant solution after working with infectious material and it is sometimes advisable to work on a towel wrung out in a disinfectant like 2 per cent lysol or tricresol solution.

5. Pipets, test tubes, instruments, etc., employed in the examination of infectious material should be placed immediately in a disinfectant solution like 2 per cent lysol or tricresol, or immediately sterilized by boiling.

6. In grinding dried bacteria a mask should be worn or the procedure conducted in a special hood insuring against the inhalation of dust.

7. Containers contaminated with sputum, feces, etc., or slides soiled with excessive amounts of vaginal or urethral pus, etc., should not be handled at all, but consigned to a disinfecting process.

8. Chemical work involving the production of irritating and dangerous fumes should be always conducted under hoods with good exhausts and adequate ventilation.

9. Electric heaters and other electrical apparatus should be inspected frequently and immediately repaired as a safeguard against short circuiting and fires. Bunsen burners should never be used around inflammable material. Ether, alcohol and the like should be carefully kept away from all possible contact.

10. All laboratory workers should be immunized against diphtheria if Schick-positive; also against typhoid and paratyphoid fevers. Cowpox vaccination is advisable every few years and after every contact with smallpox. Good general health should

be maintained at all costs as an effective means for keeping natural immunity and resistance at a high level.

### EMERGENCY TREATMENT OF ACCIDENTS

**Cuts and Needle Pricks.**—1. Remove all foreign matter, such as pieces of glass, dirt, etc.; then apply 3.5 per cent tincture of iodine, taking care that the tincture reaches all crevices of the wound.

2. If the cut is slight or does not bleed copiously, bandage, placing a small pad of gauze directly over the wound and bandaging tightly enough to stop the flow of blood. If a small cut does not stop bleeding from the pressure bandage alone, apply peroxide of hydrogen and bandage with a pad of gauze over the wound.

3. If the cut is severe and bleeds copiously, apply a tourniquet to check the bleeding. If the cut is in an artery, indicated by the blood being a bright scarlet and flowing in an intermittent stream, the tourniquet is to be placed between the cut and the heart. If in a vein, shown by dark, purplish blood, the tourniquet should be placed between the capillaries and the wound. Under no conditions should a tourniquet be allowed to remain in place for more than 2 hours at a time.

4. Needle pricks should be squeezed to promote bleeding, carefully washed with hot water and soap and treated with tincture of iodine or 1:500 metaphen or mercuraphen solutions.

**Burns.**—*From Flames or Hot Objects.*—1. Dress with butesin picrate ointment, and, if serious or covering a large area, bandage over the dressing. Dressing should be completely changed at least once a day.

2. Blisters forming from burns should be opened and drained by puncturing in at least 2 places near the edge and pressing out the liquid. The puncture may be made with a flame-sterilized needle or razor blade. When changing dressing, any blisters present should be drained again.

*From Chemical Agents.*—1. Burns from *strong acids*, bromine, chlorine, phosphorus or other material of acid character, are washed first with large quantities of water, then with 5 per cent sodium bicarbonate or 5 per cent ammonium hydroxide solution, dressed and bandaged as above.

2. Burns from *strong alkalis*, sodium hydroxide, metallic sodium or potassium or other materials of alkaline nature, are washed first with large quantities of water, then with 5 per cent boric or acetic acid solution, dressed or bandaged as above.

3. Burns from *phenol* are washed immediately with strong alcohol, then dressed and bandaged, if necessary.

*Of the Eye.*—Flush first with large amounts of water. Then, if due to *acid* material or formaldehyde, flush with 5 per cent sodium bicarbonate solution. If due to *alkaline* material, flush with 5 per cent boric acid solution. In either case a drop or two of castor, cottonseed, or olive oil in the eye is good as a soothing agent.

**Scalds.**—Blisters should be drained as above; then the injury is dressed and bandaged, using suitable neutralizing agents if due to acid or alkaline materials, as prescribed for burns.

**Swallowing of Mineral Acids.**—1. Wash out the mouth immediately with large amounts of water and alkaline solutions like decinormal sodium hydroxide, milk of magnesia, etc.



2. Give calcined magnesia, white magnesia, milk of magnesia, or lime-water *immediately*, mixed in milk or any mucilaginous fluid that will act as a demulcent. Repeat the dose at short intervals until neutralization of the acid may be inferred. Do not give carbonates as antidotes for mineral acids. Oleaginous and mucilaginous fluids should be given freely, even as vehicles for the antidotes. In the case of strong sulphuric acid, water, if given at all, should be given in large quantities, on account of the heat developed. Ice may be given to relieve thirst and pain. Stomach tube and emetics should not be used. Or, give a teaspoonful of *universal antidote* in a small glass of warm water. This antidote is made by mixing 2 parts of pulverized charcoal, 1 part of magnesium oxide and 1 part of tannic acid. It is well to keep a supply on hand.

**Swallowing of Caustic Alkalis.**—1. Wash out the mouth immediately with large amounts of water and weak acid solutions.

2. Administer a weak acid, such as 5 per cent acetic acid, vinegar, or lemon juice, until neutralization of the alkali may be inferred; or, give a teaspoonful of universal antidote (see above) in a small glass of warm water. Give butter, olive or cottonseed oil; or other oils or fats to form soaps.

3. Assist vomiting by drafts of tepid water.

**Swallowing of Phenol and Phenolic Compounds.**—1. Wash out the mouth immediately with dilute alcohol (30 to 40 per cent).

2. Give *immediately* 4 ounces of alcohol mixed with 4 ounces of water, or  $\frac{1}{2}$  pint of whisky, and remove from stomach by use of emetic, preferably of mustard (tablespoonful in enough water to make a thin cream). If stomach tube is used, it must be with great care.

**Inhalation of Corrosive Gases.**—1. Remove patient to fresh air, and place prone, face down, with head slightly lower than the chest, so that vapors may drain from the lungs.

2. Permit inhalation of vapors of acetic acid if ammonia is active agent, and of dilute ammonia if acid vapors are being treated.

3. Inhalation of vapors of alcohol or ether soothes the respiratory tract.

4. *Toxic headaches* due to the inhalation of vapors of various materials may be combated by removal to fresh air, administration of 5 to 10 grains of aspirin, and allowing the patient to rest for a time.

5. For *hydrogen sulphide* fumes inhale ammonia from 5 per cent ammonium hydroxide, or inhale fresh air containing a small proportion of chlorine. Administer milk, white of egg in water, olive or cotton seed oil, etc.

**Swallowing of Virulent Cultures.**—1. Accidental contaminations of the mouth with cultures of staphylococci, streptococci, pneumococci, etc., are not dangerous although it is advisable to immediately rinse well with hot water and disinfectant like 1:5000 metaphen, 1:2000 bichloride of mercury, diluted hydrogen peroxide, etc.

2. Virulent diphtheria cultures are much more dangerous. The above measures should be employed immediately. If Schick-negative, there is little or no danger of infection. If Schick-positive, infection may follow unless a prophylactic injection of 1000 units of antitoxin is taken.

3. The swallowing of typhoid, cholera and dysentery cultures also carries some danger, especially in the case of freshly isolated cultures. The mouth should be dis-

infected immediately as described above. In the case of typhoid cultures, it may be advisable to undergo vaccination unless this has been done within 2 years.

4. Great care is also required in working with *Br. abortus* (*Br. melitensis*). In case of contamination, immediate immunization with vaccine is recommended following the cleansing measures recommended above. Great care is also required when working with *Bact. tularensis*.

**Contamination with Syphilitic Material.**—1. In case of needle pricks or cuts while removing chancre or other syphilitic material for darkfield or other examinations, squeeze to promote bleeding; then wash carefully with *hot* water and *plenty of soap*. Dry and thoroughly apply 33 per cent ointment of calomel or metallic mercury or 1:1000 solution of bichloride of mercury (ointments preferred). Renew the applications twice a day for at least 3 days.

2. Contamination of the hands with the blood of syphilitic patients while taking blood for the Wassermann test, giving injections, etc., is rarely dangerous and not at all unless there are needle pricks, cuts or abrasions. In case of contamination of cuts and pricks with blood, it is advisable, however, to use the measures described above.

3. We have never heard of or seen syphilitic infection following accidental contamination of the mouth with syphilitic serum or cerebrospinal fluid while conducting the Wassermann and other tests. The manipulations involved in the preparation of serum and especially heating at 55° C. for 10 minutes or longer, are almost sure to kill the spirochetes, even if they were present, as *Treponema pallidum* is very susceptible to heat.

Spinal fluids, however, especially from paretics, are potentially more dangerous, particularly if contamination occurs during spinal puncture or soon thereafter. If needle pricks or cuts occur, the measures given above should be applied. If spinal fluid is accidentally taken into the mouth during total cell counts or other examinations, it will suffice to rinse thoroughly with water and 1:500 metaphen or mercurophen or 1:1000 bichloride of mercury.

# CLINICAL PATHOLOGICAL METHODS

## METHODS FOR THE EXAMINATION OF THE BLOOD

**Routine Blood Examinations.**—Next to urine examinations, it is probable that red and white corpuscle counts, hemoglobin estimations and differential leukocyte counts are the laboratory examinations conducted most frequently in clinical pathology. Cheap diluting pipets, counting chambers and coverglasses are responsible for a great deal of error; only the better grades are recommended and preferably those with the U. S. Bureau of Standards' certification. Pipets with broken tips are especially likely to be inaccurate. It is recommended that only the best apparatus be employed with scrupulous attention to all technical details. *Considerable practice and experience are required for obtaining results of acceptable accuracy.* Almost invariably variations from the normal should be carefully checked.

Routine examinations vary in different laboratories and in the practice of different physicians. These usually include the following, named in the order in which specimens of blood should be collected: (1) Hemoglobin estimation; (2) erythrocyte count; (3) leukocyte count and (4) smears or films for a differential leukocyte count and the detection of abnormal leukocytes and erythrocytes.

### APPARATUS FOR BLOOD EXAMINATIONS

1. It is advisable for the laboratory to be supplied with either the Thoma (Fig. 20) or Trenner (Fig. 21) automatic pipets for the counting of erythrocytes and leukocytes. The Thoma erythrocyte pipet gives dilutions ranging from 1:100 to 1:1000 and the Trenner a dilution of 1:200. The Thoma leukocyte pipet gives dilutions 1:10 up to 1:100 and is useful for very high and very low counts. The Trenner gives a 1:20 dilution although pipets giving a 1:10 dilution are available.

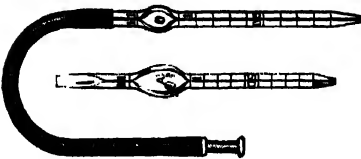


FIG. 20.—THOMA DILUTING PIPETS

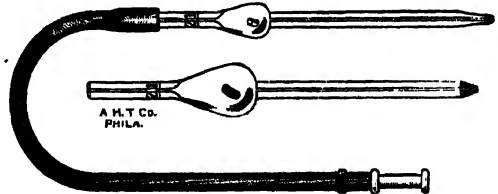


FIG. 21.—TRENNER AUTOMATIC PIPETS

2. All pipets should be guaranteed to be within the tolerance for accuracy established by the United States Bureau of Standards of  $\pm 3$  per cent for the principal interval. Pipets with the Bureau of Standards' certification are recommended, although they are probably too expensive for routine work. It is advisable, however, for every

laboratory to have at least one certified red corpuscle and leukocyte pipet with which new pipets may be compared before the latter are accepted for work.

3. While many different counting chambers are in use, the Levy with the improved Neubauer ruling (Fig. 22) is recommended; also the Levy-Hausser with bakelite holder and double Neubauer ruling. At slight extra cost these may be purchased with the United States Bureau of Standards' certification and every laboratory should have at least one for comparative counts with new chambers before the latter are adopted for routine work.

The Spencer Lens Company offers a "bright line" counting chamber in which the ruling is made on a metallic coating on the glass. In focus, the lines appear white and are easily visible; it is also stated that the nature of the surface results in a more even distribution of cells.

4. Accurate blood cell counting requires a coverglass with optically plane surfaces. *The curvature of an ordinary coverglass as used in bacteriological work is such as to render an otherwise perfect counting chamber absolutely useless from the standpoint of accuracy.*

For very little extra cost, coverglass may be obtained with the United States Bureau of Standards' certification, and these are recommended for the best work. The Hausser reenforced precision glasses are recommended for the counting chamber method of standardizing vaccines.

5. The Marbel blood cell calculator (Fig. 23) is highly recommended for red and white corpuscle counting and for differential leukocyte counting. The Veeder hand counter, while not as elaborate, is less expensive and very useful (Fig. 24).

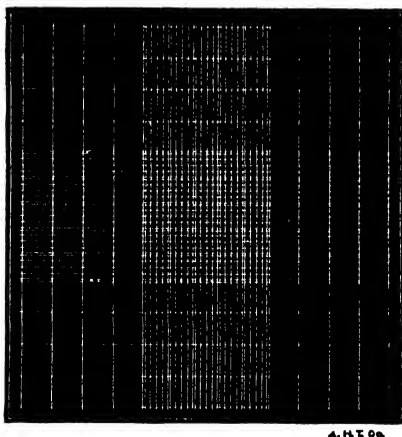


FIG. 22.—ENTIRE AREA OF IMPROVED NEUBAUER RULING

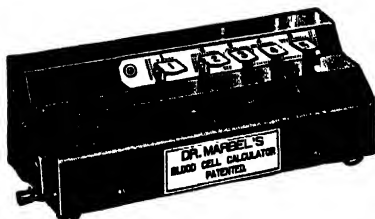


FIG. 23.—MARBEL BLOOD CELL CALCULATOR



FIG. 24.—THE VEEDER HAND COUNTER

## METHODS FOR CLEANING BLOOD APPARATUS

1. Pipets and counting chambers should be cleaned immediately or as soon as possible after using.
2. Draw water through pipets until all traces of blood and serum are removed.
3. Without drying, draw through alcohol or acetone.

4. Draw through ether (may be omitted if acetone is used).
5. Draw air through until the pipet is dry (if properly dried the bead in the bulb should be freely movable).
6. If blood has dried in the stem of a pipet, remove it with horsehair or fine stiff wire and fill pipet with antiformin, nitric acid or bichromate-sulphuric acid cleaning fluid and allow it to stand overnight; then clean thoroughly as described. The use of undiluted antiformin for 20 minutes is usually sufficient.



FIG. 25.—MULTIPLE ASPIRATING NOZZLE

7. The multiple aspirating nozzle for attachment to a pump (Fig. 25) or the Haden cleansing apparatus (Fig. 26) is very convenient and time-saving for the simultaneous cleaning of 1 to 4 pipets.

8. Immediately after use, the ruled area, the surface of the slide, and the coverglass of the counting chamber should be cleaned with water and dried with a soft lint-free cloth. If this is not done the lines will become partly obliterated with debris. If diluted blood has been allowed to remain on the slide or the ruling becomes indistinct, it may be necessary to immerse the slide in decinormal sodium hydroxide or in one of the solutions mentioned above for cleaning pipets. The Levy-Hausser chamber may also be washed with alcohol and ether without damage.

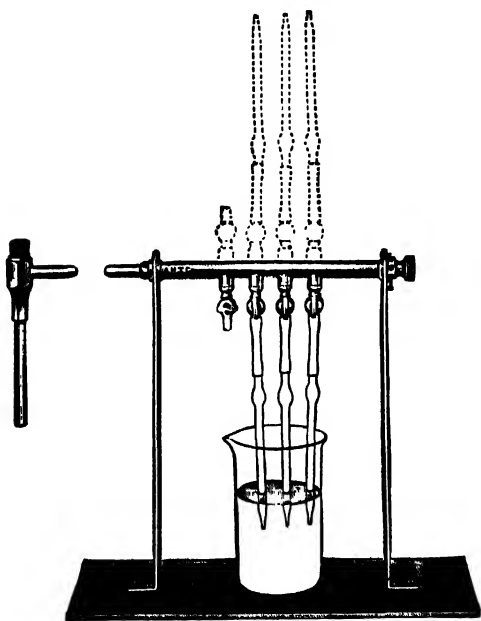


FIG. 26.—HADEN CLEANSING APPARATUS



FIG. 27.—HAGEDORN NEEDLE IN CORK FOR FINGER OR EAR PUNCTURE

### METHODS FOR OBTAINING CAPILLARY BLOOD

1. Blood is usually obtained by pricking a finger or the lobe of an ear. In the case of babies, the big toe or heel may be employed. Otherwise, the finger is always to be preferred because pipets can be used more accurately, the technician need not stand, and hair is not disarranged. If, however, it is necessary to employ the ear,

the puncture should be made in the edge of the lobe. The ear upon which the patient has been lying should not be used because of congestion.

2. All apparatus (hemoglobinometer, pipets, diluting solutions, slides or cover-glasses), should be in readiness, preferably on a tray.

3. Probably the best "sticker" is a straight Hagedorn needle placed in a cork and kept in a bottle of 95 per cent alcohol (Fig. 27); 70 per cent alcohol will rust the needle. A sharp needle should always be employed. This needle makes a triangular puncture which bleeds freely; an ordinary round needle or pin is unsatisfactory. Simple blood lancets (Fig. 28) and spring lancets are available. Care should be exercised when using the latter; otherwise the incision may be too deep.



FIG. 28.—A SIMPLE BLOOD LANCET

4. The finger must be free from edema, congestion and inflammation. Do not use the finger of a hand which has been hanging over the side of the bed as it is likely to be congested. The finger should be warm and the circulation free. If the hand is cold and clammy, too much squeezing is required with resulting error due to tissue juices. Under these circumstances immersion of the hand in warm water for a few minutes, or vigorous massage, is required.

5. The side of the last phalanx of the middle finger is preferred (Fig. 29). If the tip of a finger is used, lance across rather than parallel with the lines of the skin.

6. Cleanse the skin by rubbing vigorously with a cotton pledget saturated with



FIG. 29.—PRICKING SIDE OF FINGER WITH HAGEDORN NEEDLE

alcohol. Use dry cotton or gauze to dry the skin. *Do not prick a wet finger* as the alcohol will cause the blood to spread out in a thin layer instead of collecting in a large drop; alcohol will also coagulate the proteins of the blood, making it impossible to draw the blood into pipets.

7. Squeeze the tip of the finger and make a firm, quick, deep puncture so that bleeding occurs on slight pressure. *Always wipe away the first drop of blood.* Use light pressure, if necessary, to produce succeeding drops.

8. When the necessary specimens have been secured, wipe the finger with alcohol and have the patient hold an alcohol pledget on the finger until all bleeding has stopped.

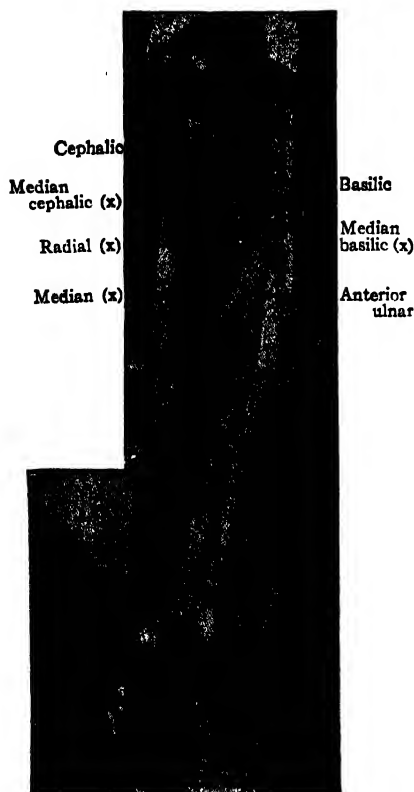


FIG. 30.—CHOICE OF VEINS FOR SECURING BLOOD

The sites of choice are marked with an (x); it is always advisable to avoid punctures near the crease of the elbow in order to avoid subsequent discomfort. (From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

## METHODS FOR OBTAINING VENOUS BLOOD

1. In the great majority of cases the veins of the forearm, especially those about the elbow, are employed, *i.e.*, the median cephalic median basilic, common ulnar and radial veins (Fig. 30). The median, ulnar and cephalic veins are also employed; indeed, any prominent, *well supported* vein may be used, but the median basilic is probably the least desirable for the inexperienced because of the proximity of the internal cutaneous nerve and because it is separated from the brachial artery only by the biceps fascia which may be pierced by clumsy technic.

The most prominent vein is not always the easiest to enter because it may roll under the needle; under such circumstances it is especially advisable to use a short and very sharp needle. The best veins are those which are full, but well supported by the subcutaneous tissues to prevent rolling and dimpling during the introduction of the needle.

In some cases the veins about the wrists or on the back of the hand may be employed; however, never by choice but only by necessity, since they are small, very thin, easily pierced and likely to give subcutaneous hemorrhages.

The same is true of veins about the ankle. When these veins are employed the needle must be short, very sharp and not larger than gauge No. 20 or 22.

2. The practical importance of a proper tourniquet is not to be overlooked. It should be at least an inch wide in order to produce the minimum of discomfort and should be applied in such a manner that it may be released instantly and with no commotion.

In many instances a tourniquet may be improvised by twisting a towel, a piece of bandage or the shirt sleeve, and held by an assistant or the patient, but these procedures are inferior to a piece of ordinary garter elastic or a strip of rubber sheeting held by a slip knot. Sometimes an assistant, or even the patient, may firmly grasp the arm and distend the vein sufficiently. The ordinary surgical tourniquet is too clumsy and is generally unsatisfactory; the tourniquet of rubber tubing held by a hemostat is only a makeshift, apt to wrinkle and pinch the skin. The cuff of a sphygmometer makes a most excellent tourniquet, being broad and comfortable and readily applied and released. A satisfactory tourniquet consists of 15 inches of good garter elastic, about an inch wide, applied with one firm turn and held by a slip knot (Fig. 31) which is released instantly by a slight pull without disturbance. The tourniquet should be applied flat without wrinkling or pinching the skin.



FIG. 31.—SHOWING THE DETAILS OF A SATISFACTORY SLIP KNOT TOURNIQUET OF ORDINARY GARTER ELASTIC

(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

3. After applying the tourniquet the patient should open and close the hand vigorously for a few seconds and keep the hand clenched during the introduction of the needle; this aids in distending the superficial veins.

The tourniquet should not be applied too soon; all should be in readiness, as the most comfortable tourniquet becomes very uncomfortable when venous stasis is maintained over several minutes. *The tourniquet should be released if more than 2 minutes are required for securing blood.*

4. For simple venous puncture it is sufficient to prepare the skin by cleansing with 1:1000 mercuraphen or bichloride of mercury in alcohol; tincture of iodine (5



per cent) or a solution of picric acid (5 per cent) may be applied, but if so, they should be wiped away with alcohol so as not to hide the vein.

If it is necessary to incise the skin, there should be thorough preliminary cleansing with soap and water, followed by iodine and a final cleansing with alcohol in order to guard against infection of the skin and to prevent scar formation as much as possible.

5. The vein should be steadied with the thumb and index finger; some physicians prefer to slightly stretch the skin over the vein to steady it although this tends to flatten the vein and increase the chances of transfixion. Various clamps have been devised for grasping and steadying the vein through the skin, but there is no better means than the finger and thumb of the disengaged hand.

6. The needle should be held at an acute angle to the arm (Fig. 32); it is a mistake to attempt entering the vein at a right angle because of the danger of passing



FIG. 32.—CORRECT ANGLE FOR HOLDING A SYRINGE FOR ENTERING A VEIN

(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

through the vessel. It is also good practice to puncture the vein obliquely, that is, with the needle directed to the side of the vein rather than directly over its top and especially if the vessel is very prominent, sclerotic and freely movable. When an attempt is made to puncture directly over the vein the needle sometimes slips over its top and passes into the perivascular tissues. However, when the vein is not prominent, but is felt full and elastic in the subcutaneous tissues, one may enter the needle directly over the vein as the vessel is well supported and unable to roll.

*With a Syringe.*—A sterilized 5 or 10 cc. Luer or Record syringe, fitted with a No. 20 to 22 needle, is recommended. It is not necessary to use a larger needle which inflicts unnecessary pain. The needle should be short and not over  $1\frac{1}{4}$  inches; long needles interfere with the flow of blood and are more difficult to enter into a vein. The bevel must be short rather than long, but not too stumpy; it is imperative to use

a sharp and rust-free needle to conduct the operation with the minimum of pain and leave no stained puncture site in the skin.

**With a Needle.**—Blood may be secured from a vein by merely introducing a sufficiently large sterilized needle and allowing the blood to flow into a prepared test tube or vial. For this purpose the needle should be gauge No. 18 to 20 or even larger since suction is not applied and the shaft should not be too long as otherwise coagulation may occur. It is of advantage to grasp the needle with a hemostat as shown in Figure 33. This method, however, is apt to be "messy" and is inferior to the Keidel tube and syringe methods.

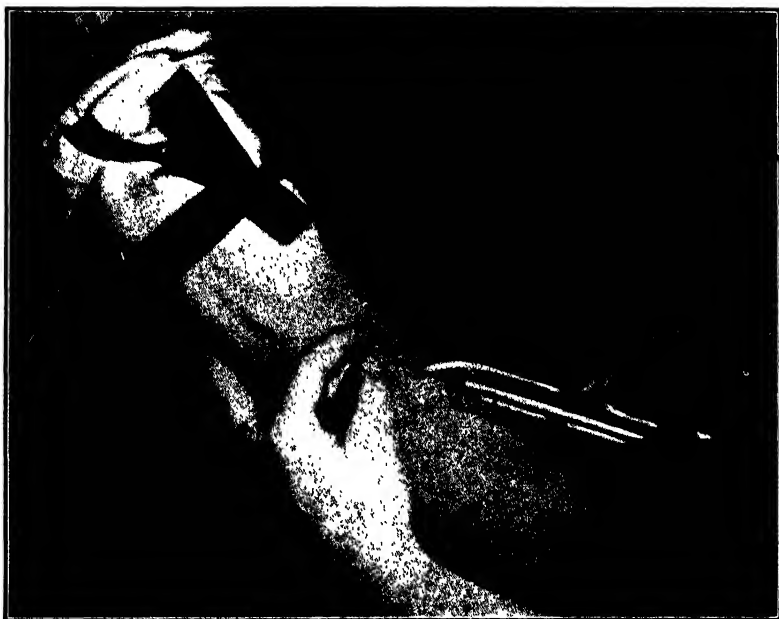


FIG. 33.—METHOD OF WITHDRAWING BLOOD WITH A NEEDLE  
(From Kolmer in *Keen's Surgery*, W. B. Saunders Co., Philadelphia.)

**From Infants and Children.**—1. Usually in children over 6 years of age, blood may be obtained from an arm vein. In younger children and infants the external jugular vein (Fig. 34) or a temporal vein may be used.

2. From infants under 1 year of age the superior longitudinal sinus may be employed as follows: (a) The infant is wrapped in a blanket and the head is steadied by an assistant (Fig. 35); (b) the puncture is made on the median line of the posterior angle of the anterior fontanelle; (c) the skin is carefully cleansed. The needle, gauge No. 18, with a short bevel, sterilized and attached to a sterile 5 cc. Record or Luer syringe is passed inward at a right angle for a distance of about 4 mm. and suction made; if blood does not flow, the needle should be passed about 2 mm. farther, which suffices for the majority of children up to 15 months of age; (d) at least 3 to 5 cc. of blood may be withdrawn safely. The puncture site is then cleansed and may be sealed with a touch of collodion.



FIG. 34.—METHOD OF OBTAINING BLOOD FROM AN EXTERNAL JUGULAR VEIN  
(From Kolmer in *Keen's Surgery*, W. B. Saunders Co., Philadelphia.)



FIG. 35.—METHOD OF OBTAINING BLOOD FROM THE SUPERIOR  
LONGITUDINAL SINUS  
(From Kolmer in *Keen's Surgery*, W. B. Saunders Co., Philadelphia.)

### METHOD OF USING OXALATED BLOOD FOR HEMATOLOGICAL EXAMINATIONS

According to Osgood, Haskins and Trotman<sup>1</sup> oxalated blood may be used for the following examinations if the latter are made within the designated time after the collection of blood:

	<i>Hours</i>
Hemoglobin estimation .....	24
Red cell count .....	24
Platelet count .....	1
Red cell volume .....	3

Color index .....	24
Volume index .....	3
Saturation index .....	3
Icterus index .....	4
Van den Bergh test .....	4
White cell count .....	24
Smear for differential count .....	1
Peroxidase test .....	3
Fragility test .....	3
Sedimentation rate .....	3
Reticulocyte count .....	24

1. A mixture of dry ammonium and potassium oxalate is advised <sup>2</sup> which may be prepared by dissolving 1.2 gm. of dry ammonium oxalate and 0.8 gm. of dry potassium oxalate in 100 cc. neutral distilled water. Place 0.5 cc. in each of a series of chemically clean dry test tubes stoppered with cotton and evaporate to dryness in a hot air sterilizer. These amounts of the 2 oxalates are for 5 cc. of blood.

2. Draw exactly 5 cc. of blood from a vein as described.

3. *Remove the needle* from the syringe before discharging the blood into oxalate tube as hemolysis may occur if the blood is forced through the needle.

4. Stopper test tube (never use cotton) and shake at once by holding test tube obliquely in left hand and tapping other end with right hand.

5. The blood must be thoroughly mixed in this manner *immediately* before samples are withdrawn for any test.

6. Samples should be taken directly from the test tube—not from blood poured out on a slide or watch glass.

7. The tube must be kept stoppered at all times when not in use and in case of delay should be kept in a refrigerator.

8. The time limits given above should be observed if the most accurate results are desired although, as a rule, a slightly longer time will not introduce clinical error.

The advantages of this method are that if during the study of the blood further hematological work is deemed desirable, it may be done on the same sample. Repeated estimations are possible for checking results. If an unusual or interesting blood picture is encountered, as many slides as are desirable may be made for future reference or for teaching purposes without again disturbing the patient which is very convenient.

## NORMAL VALUES AND RANGES

Normal values and ranges vary according to age, sex, locality, etc., and the methods employed. Those summarized in Tables 2 and 3 are believed to be fairly representative for normal healthy human beings under average conditions. It is recommended that blanks employed for submitting reports on hematological examinations always state the normal values or ranges for the assistance thereby given clinicians in the interpretation of results. Table 4 gives normal values and ranges for the domestic animals commonly employed for laboratory purposes.

**TABLE 2.—NORMAL VALUES AND RANGES FOR HUMAN BEINGS**

Constituents	Normal
Total Erythrocytes (per c.mm. of blood)	Under the best of conditions, at least 5 per cent error in counting, increased by physiologic causes: (a) Muscular activity; (b) psychic factors (excitement or fear); (c) high altitudes, and (d) sex <i>after puberty</i> (higher in males than females). Meals have no influence except reduction through high fluid intake. Climate, temperature and season have no influence except possible increase from dehydration (profuse sweating with low fluid intake). <i>Age has an important influence.</i> Infants 6 million at <i>birth</i> dropping in 2 weeks to childhood level 4 to 5.5 million. Adult males 4.5 to 6 million. Adult females 4.2 to 5.4 million.
Reticulocytes	0.5 to 1.5 per cent of erythrocytes.
Volume packed erythrocytes. (cc. per 100 cc. of blood)	Infants 60, falling to childhood level 44 to 32. Adult males 39 to 52. Adult females 35 to 48.
Hemoglobin—Gms. per 100 cc. (Equals vols. per cent oxygen capacity ÷ 1.34)	Infants 21 to 27 at birth, falling in first 2 months to childhood level 18 to 10. Adult males 14 to 17. Adult females 12 to 16.
Mean corpuscular volume (cubic microns)	Infants 105 at birth falling rapidly to childhood level 87 to 73. Adults 82 to 92.
Mean corpuscular hemoglobin in micrograms ( $10^{-12}$ gm.)	Infants about 40 at birth falling rapidly to childhood level 33 to 27. Adults 29 to 31.
Mean corpuscular hemoglobin concentration (gms. per 100 cc. of packed cells)	Infants about 45 at birth falling rapidly to childhood level 41 to 34. Adults 33 to 37.
Color Index Volume Index Saturation Index	See text on page 66.
Sedimentation Rate (1 hour)	Males 0 to 8 mm. Females 0 to 10 mm.

**TABLE 2.—NORMAL VALUES AND RANGES FOR HUMAN BEINGS (Contd.)**

Fragility of Erythrocytes	Min. resistance (slight hemolysis); 0.40 to 0.46 per cent NaCl sol. Max. resistance (complete hemolysis); 0.30 to 0.36 per cent NaCl sol.
Total Leukocytes (per c.mm. of blood)	Infants 10,000 or higher. Children 5,000 to 14,000. Adults 5,000 to 10,000.
Kinds of Leukocytes	(a) Immature (nonfilamented or nonsegmented) neutrophils; (b) mature (filamented or segmented) neutrophils; (c) eosinophils; (d) basophils; (e) lymphocytes and (f) monocytes. Absolute numbers and percentages vary with age. (See Table 3.)
Platelets	Vary according to different methods; in general terms 250,000 to 500,000 per cmm.
Coagulation Time	Lee and White method—5 to 8 minutes. Capillary Tube method—1 to 7 minutes. Howell method—10 to 30 minutes.
Bleeding Time	1 to 3 minutes (method of Duke).
Prothrombin Time	12 to 13 seconds (method of Quick).
Clot Retraction Time	Begins 1 hour—marked in 18 hours.

**TABLE 3.—KINDS OF NORMAL LEUKOCYTES**

	<i>Adults</i>		<i>Children**</i>	
	Per Cent *	Absolute *	Per Cent *	Absolute *
Neutrophils	60-70	3000-7000	40-65	2000-8000
Eosinophils	1-4	50-400	1-5	25-700
Basophils	0-0.5	0-50	0-0.5	0-75
Lymphocytes	20-30	1000-3000	30-60	2500-9000
Monocytes	2-6	100-600	0.5-7	25-700

*Note.*— \* Per cent and absolute numbers per c.mm. are based on normal total counts of 5,000 to 10,000 for adults, and 5,000 to 15,000 for children.

\*\* These ranges are for children between 3 months and 15 years of age. The higher counts in children are due chiefly to lymphocytes which are high in the very young and gradually decrease until counts approximate those of adults somewhere between 10 and 15 years of age. (See Table 7 on page 91.)

**TABLE 4.—NORMAL VALUES AND RANGES IN THE DOMESTIC ANIMALS \***

Species	Erythrocytes (millions)	Hemoglobin (gms. per 100 cc.)	Erythrocyte Volume (%)	Platelets (thousands)	Leukocytes (thousands)	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Horse	5.7-8.7	10.5-16.4	30-44	35-500	5-11	3,000-6,900	50-600	0-100	1,200-4,800	100-1,450
Cattle	5.0-10.3	8.0-14.5	30-50		5-12	1,200-4,800	180-1,800	0-100	2,700-6,900	150-1,800
Sheep	8.0-14.7	9.0-14.5	40-50		4-10	1,000-4,500	50-700	0-200	2,500-7,000	50-800
Swine	5.0-9.0	9.0-16.8		100	7-20	2,400-10,000	50-200	0-800	3,200-12,000	50-2,000
Goat	9.0-19.0	12.7-14.2			5-14	2,100-3,350	0-1,100	0-600	2,100-11,250	50-600
Dog	5.5-8.8	12.5-17.3	40-58	250-450	5-20	3,600-15,000	100-2,000	0-400	600-6,000	100-2,400
Cat	7.0-10.0	8.0-13.8			5-40	3,000-24,700	100-3,800	0-150	1,200-15,200	50-5,700
Monkey	5.0-7.0	12.3-19.9		155-424	7-25	2,400-12,500	50-1,250	0-100	3,200-13,750	50-3,000
Rabbit	4.5-7.0	10.4-15.6		200-1,000	3-12	1,200-6,500	0-200	50-300	1,200-6,500	50-2,000
Guinea-pig	4.5-6.8	15.6-17.3		200-900	5-23	1,800-10,000	100-3,000	0-400	2,100-11,000	50-4,000
Rat	7.0-10.0	15.6-19.0		500-1,200	6-16	1,200-6,000	0-600	0-200	4,000-12,000	150-550
Mouse	8.0-11.0	15.6-17.3			3-22	600-10,000	0-550	0-100	2,200-15,000	50-1,650
Chicken	1.6-4.5	8.6-16.5		45-55	16-40	4,000-16,000	400-4,000	200-1,600	8,000-24,000	1,000-6,000
Frog	0.4-0.6				10-38	700-2,600	2,600-10,000	700-2,600	6,000-22,800	

\* Compiled by A. H. Craige, Jr., V.M.D., School of Veterinary Medicine, University of Pennsylvania.

**METHODS FOR THE ESTIMATION OF HEMOGLOBIN**

**Principles.**—1. None of the methods in common use can be recommended from the standpoint of absolute accuracy. Owing to a variety of methods and standards, expressing hemoglobin in per cent may lead to confusion in interpretation. Widely varying results may be reported on the same individual examined in different laboratories. *This source of inaccuracy and misunderstanding can be overcome by expressing the results as grams of hemoglobin per 100 cc. of blood* on the assumption that volumes per cent oxygen capacity  $\div 1.34$  equals grams of hemoglobin per 100 cc.

2. The most accurate methods available at present are that of Van Slyke for determining the oxygen capacity of the blood, and that of Wong for determining the iron content. However, these are not sufficiently simple for routine work, but are valuable for checking the accuracy of clinical methods.

3. *It is recommended that reports show hemoglobin in grams per 100 cc. of blood instead of in per cent.* The average normal values at various ages in relation to sex may be taken as follows (grams per 100 cc. of blood):

Age	Male	Female
Birth .....	23 .....	Same
10 days .....	21.4 .....	"
1 month .....	19.0 .....	"
2 months .....	16.2 .....	"
6 months .....	13.4 .....	"
1 year .....	12.6 .....	"
3 years .....	13.1 .....	"
7 years .....	14.0 .....	"
15 years .....	15.4 .....	15.0
20 years .....	16.8 .....	15.4
60 years .....	16.9 .....	15.9
80 years .....	15.7 .....	15.1

4. Grams of hemoglobin per 100 cc. of blood may be converted into percentage as follows: Divide the normal in grams according to age and sex into 100 to obtain the factor; multiply the grams observed by the factor. Example:

Male, age 20 years

Normal hemoglobin: 16.8 gms. per 100 cc.

100 divided by 16.8 = 5.95 the factor

Hemoglobin determination: 14.3 gms. per 100 cc.

$14.3 \times 5.95 = 84$  per cent hemoglobin

5. Normal ranges may be taken as follows (grams per 100 cc. of blood):

Age	Minimum	Maximum
1 to 13 days	17	25
14 to 60 days	11	18
3 to 5 months	10	15
6 months to 3 years	9	14
4 to 15 years	10	14
Adults—Females	12	16
Males	14	18



7. For general purposes grams of hemoglobin per 100 cc. of blood may be converted into percentage by multiplying the number of grams by 6.

**Haden-Hausser Method.**—This method is recommended for routine use because of its simplicity and accuracy. An inexpensive clinical model of the hemoglobinometer is suitable for clinical work (Fig. 36). A larger and more expensive laboratory model

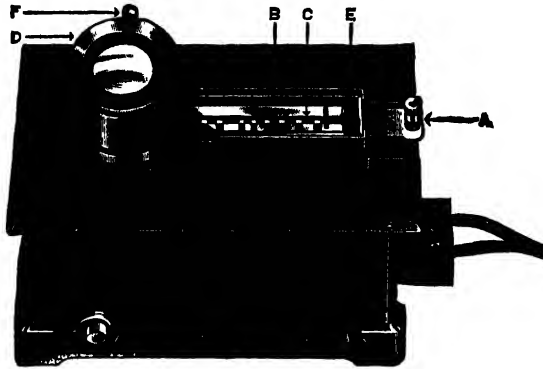


FIG. 36.—CLINICAL MODEL OF THE HADEN-HAUSSER HEMOGLOBINOMETER

A, Movable carrier; B, Comparator slide; C, Coverglass; D, Reading microscope; E, Wedge-shaped channel; F, Shutter.

with a special lighting arrangement is also available. Hemoglobin is converted into acid hematin and the color compared with a permanent color scale of tinted glass as follows:

1. Draw blood into a leukocyte pipet to the 0.5 mark and decinormal hydrochloric acid to the 11 mark, giving a 1:20 dilution. If the hemoglobin is low, use a 1:10 dilution (blood up to 1.0 mark) and divide the final reading by 2.

2. Allow the pipet to stand about 30 minutes for the conversion of hemoglobin into acid hematin.

3. Assemble the comparator slide and coverglass by applying the coverglass with beveled side down and with the lower and left edges in contact with the metal frame. Place in the comparator.

4. Mix the pipet thoroughly and discard 1 or 2 drops.

5. Run the solution into the wedge-shaped dilution channel of the comparator which fills by capillary attraction.

6. Compare with the standard through fenestrae (in lower half of slide) in the frame which holds the cell and standard. Move the comparator through the field of the reading microscope and match the colors. On the scale there is a total range of from 7.5 to 19 grams of hemoglobin per 100 cc. of blood.

**Wintrobe Method.**—This is also an acid hematin method employing the Hellige-Wintrobe hemometer (Fig. 37). Blood from the finger is diluted in an erythrocyte pipet with decinormal hydrochloric acid. The standard is a yellow glass and 14.5 grams equal 100 per cent. Directions for use are supplied with the instrument.

**Photoelectric Colorimeter Method.**—This is an accurate method first introduced for routine work by Sanford, Sheard and Osterberg.<sup>3</sup> There are now several instruments available. These instruments may be used for many other determinations in

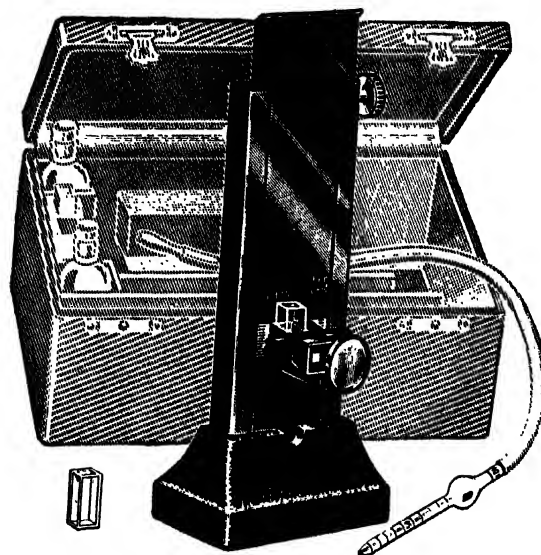


FIG. 37.—THE HELIGE-WINTROBE HEMOMETER

addition to hemoglobin. There is no attempt to match colors; therefore, subjective errors are avoided. With the Klett-Summerson Colorimeter (test tube model) the technic is as follows:

1. Place 5.0 cc. of N/10 hydrochloric acid in a colorimeter tube.
2. Collect 0.02 cc. of blood in a pipet calibrated to contain that amount.
3. Remove excess blood from the outside of the pipet and then deliver the blood into the hydrochloric acid solution in the colorimeter tube. Rinse out the pipet by repeatedly drawing up and blowing back portions of the liquid in the tube, fixing the contents of the tube well with the tip of the pipet at the same time.
4. Remove pipet and allow tube to stand at room temperature for at least 30 minutes, to permit maximum color development.
5. Read in the colorimeter against a distilled water 0.
6. Reading of unknown  $\times$  hemoglobin factor = gm. per cent hemoglobin.
7. The factor is obtained from the scale reading for a blood with known hemoglobin content. Procure a sample of fresh blood and determine its hemoglobin content by oxygen capacity, carbon monoxide capacity, or by the Wong methods. Run a colorimetric determination as described above on duplicate or triplicate 0.02 cc. portions of this blood.

From the hemoglobin content and the scale readings (averaged) the calibration factor is determined as follows:

$$\frac{\text{Gm. per cent hemoglobin in blood}}{\text{Average scale reading}} = \text{Hemoglobin factor.}$$

**Sanford, Sheard and Osterberg Method.**—This is an accurate method, but not widely used for clinical purposes; the photometer is expensive.

1. Dilute 0.1 cc. of blood in 20 cc. of 0.1 per cent solution of sodium carbonate,

thus making a 1:200 solution of oxyhemoglobin. This is most conveniently and most accurately done by making the dilutions from a sample blood immediately after it has been obtained by venipuncture.

2. The photometer has a green glass filter in front of the photronic type of photo-electric cell. This filter transmits light in its maximal intensity at that portion of the spectrum where the maximal absorption occurs in one of the oxyhemoglobin bands. The light intensity through a standard spectroscopic cell, which is filled with 0.1 per cent sodium carbonate, is first adjusted with an iris diaphragm so that the reading on the meter is 100.

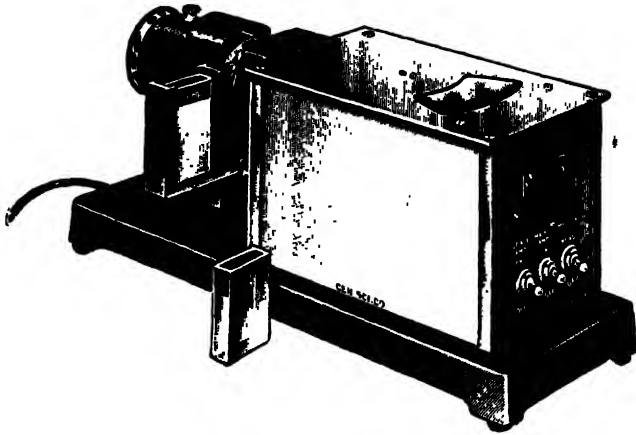


FIG. 38.—THE CENCO-SHEARD-SANFORD PHOTOMETER

3. The specimen of diluted blood is placed in a similar spectroscopic cell, is then moved into the path of light in the carrier, and the reading is made on the meter (Fig. 38).

4. This lower reading really represents the decrease in current from the photronic cell which is the result of the light absorption of oxyhemoglobin in the green portion of the spectrum. This *reading is translated directly* into a value for grams of hemoglobin per 100 cc. of blood by referring to a chart which is prepared individually for each instrument by the manufacturer, based on oxygen-capacity determinations.



FIG. 39.—THE SAHLI HAEMOMETER

This photometer has proven very satisfactory, especially in laboratories in which a large number of routine determinations are made. Its only disadvantage is the cost, which is somewhat more than a colorimeter. However, the instrument may be used as a colorimeter or a nephelometer; in fact there are many uses for it other than as a hemoglobinometer. The method is rapid and accurate. There is no attempt to match colors; therefore, subjective errors are avoided.

**Sahli Method.**—1. Fill the graduated tube of Sahli's hemometer with decinormal solution of hydrochloric acid to the mark 10 (Fig. 39).

2. After cleaning finger tip or lobe of ear with alcohol, make a puncture with lancet and gently compress (*the blood should flow freely*); wipe off first drop, then fill pipet to the mark 20 with blood.

3. Immediately place the blood in the graduated tube containing the hydrochloric acid solution. Remove the last trace of blood by drawing the solution up in the pipet and expelling several times.

4. Dilute the mixture in the graduated tube with water until it has the same tint and intensity of color as the standard tube. This is done best by adding a few drops at a time and comparing after each addition.

5. When both tubes are of the same color, note the figures on the tube at the *bottom of the meniscus*.

6. The newer tubes show both per cent and grams of hemoglobin per 100 cc. of blood.

### METHOD FOR COUNTING ERYTHROCYTES

**Principles.**—1. Blood is diluted exactly 1:200 with a special pipet, using an isotonic diluting fluid for the preservation of the corpuscles. The diluted blood is then placed in a special counting chamber and the cells in a measured volume are counted. This figure is multiplied by the appropriate factor to obtain the number of erythrocytes in 1 c.mm. of undiluted blood. This procedure constitutes the method used to report the results of the erythrocyte count.

2. Leukocytes are likewise present and may be distinguished from erythrocytes, but as a general rule they are included in the count; the number present, however, is ordinarily so small that the inaccuracy is of little or no importance.

3. A pathological decrease is called *oligocythemia* or *anemia*; an increase is called *polycythemia*.



FIG. 40.—TAKING UP BLOOD INTO PIPET FROM PATIENT'S FINGER

**Procedure.**—1. Draw blood up exactly to the 0.5 mark of the Thoma pipet marked 101 (Fig. 40). If the Trenner automatic pipet is used, draw blood by suction until the stem is nearly full and then discontinue suction and allow the blood to auto-

matically reach the extremity. The operator's hand is steadied by touching a finger against the finger of the patient.

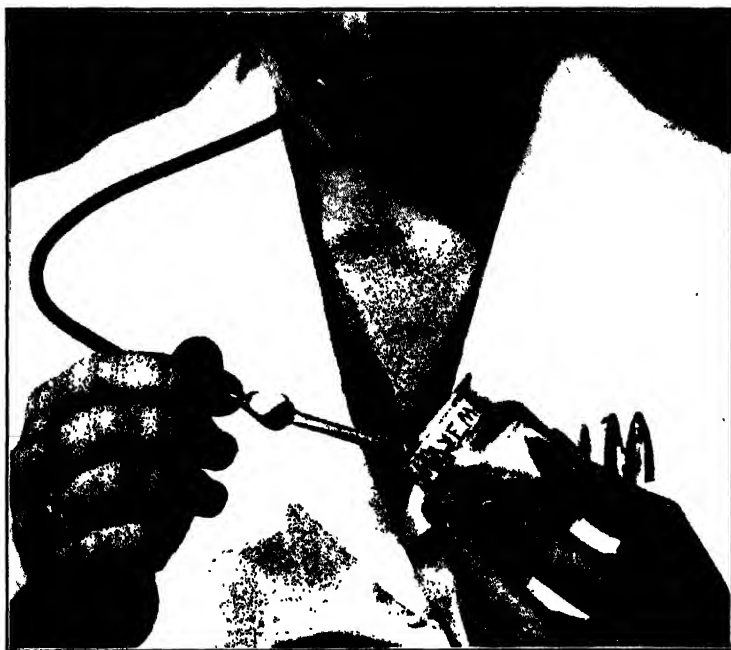


FIG. 41.—FILLING PIPET WITH DILUTING FLUID

2. Immediately draw up diluting fluid to the mark 101, thus making a dilution of 1:200 in either pipet, while rotating the pipet between the thumb and forefinger (Fig. 41). The pipet is held so that the marks are plainly visible to the eye. Use 0.85 per cent saline solution or either of the following (Hayem's preferred):

#### HAYEM'S DILUTING FLUID

Water (distilled) .....	200.0 cc.
Sodium chloride c.p. ....	1.0 gm.
Sodium sulphate (crystals) .....	5.0 gm.
Mercuric chloride .....	0.5 gm.
Carbol-fuchsin to give pink color	

#### TOISSON'S DILUTING FLUID

Neutral glycerin .....	20.0 cc.
Water (distilled) .....	160.0 cc.
Sodium sulphate (crystals) .....	8.0 gm.
Sodium chloride c.p. ....	1.0 gm.
Methyl violet .....	0.025 gm.

3. The diluting fluid should be crystal clear and filtered, if necessary, to be free of sediment.

4. If the pipet is to be carried any considerable distance to the laboratory,

stretch a broad rubber band over the ends or use one of the special closing devices (Fig. 42) to prevent the contents from escaping.

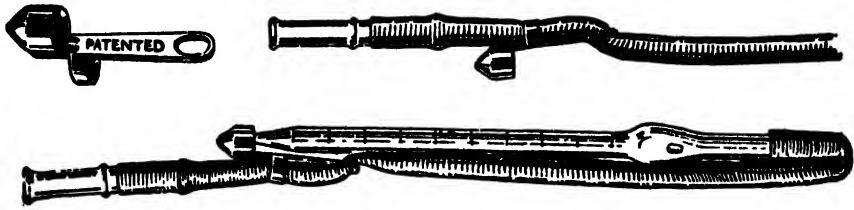


FIG. 42.—THE DRUMMOND PIPET HOLDER

5. The ruled area of the counting chamber and the coverglass must be carefully cleaned and absolutely free from dust or lint.

6. Place the coverglass in position over the ruled area, using gentle pressure to insure accurate adjustment. The Levy-Hausser chamber is provided with a pair of clips to prevent any movement during the count. While continuing pressure on the coverglass, slide the centrally placed clip into position simultaneously.

7. Close the tip of the pipet by means of the thumb. Sharply kink the rubber tubing over the other end and place the second finger over the kinked tubing. Trenner pipets are more fragile than the Thoma pipets and when filling, cleaning or attaching rubber tubing, the capillary stem should be held between the thumb and forefinger to avoid strain on the bulb. Rotate the pipet well for about a minute, holding in a horizontal position, and finally shake sidewise.

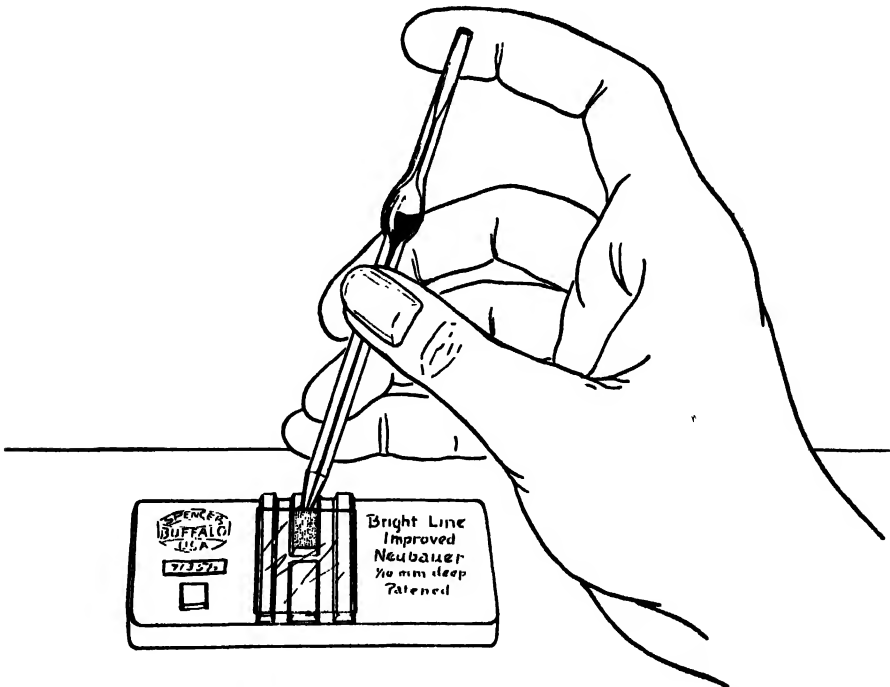


FIG. 43.—METHOD FOR FILLING COUNTING CHAMBER BY CAPILLARY ATTRACTION

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

8. Expel the fluid from the stem of the pipet and *without loss of time* touch a drop to the end of the polished surface bearing the ruling, allowing the drop to flow under the coverglass (Fig. 43). The suspension should not flow into the moats on either side, nor should any bubbles form under the coverglass.

9. Allow about 3 minutes for the corpuscles to settle.

10. Examine with 4 or 16 millimeter objective. Center the light and reduce its volume by lowering the condenser and partially closing the diaphragm.

11. Locate the finding line which leads to the ruled-off area. *Carefully avoid touching the coverglass with the lens, as this would disturb the corpuscles and lead to error in the count.*

12. When correctly focused, the corpuscles are sharply defined and the rulings appear as well-defined *black* lines (Fig. 44). When incorrectly focused, the ruled furrows appear as *white* lines and the corpuscles, which lie above the plane of the ruled surface, are out of focus (Fig. 45).

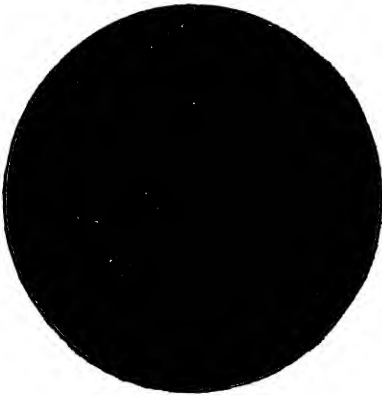


FIG. 44.—CORRECT FOCUSING

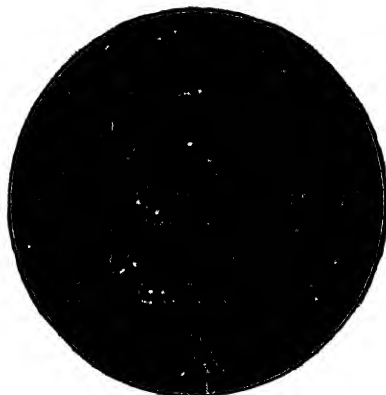


FIG. 45.—INCORRECT FOCUSING

If, however, the Spencer Lens Company "bright light" counting chamber is employed the lines will appear bright or white when in focus.

13. The counting slide will be found to have a number of small squares marked upon it. The size of these small squares is 0.05 millimeter by 0.05 millimeter or 0.0025 sq.mm. When the cover slip is in place there is a chamber formed measuring 0.1 millimeter in depth. Therefore, the small squares correspond to volumes of 0.00025 c.mm. ( $0.05 \times 0.05 \times 0.1$  millimeter = 0.00025 c.mm.).

14. Count the number in 80 of the small squares. It will be noted that the small squares are separated into groups of 16. Five of these groups, therefore, contain 80 small squares (Fig. 46). Do not count those cells touching the lower and right-hand lines, but include all touching the upper and left-hand lines.

15. Divide the number of cells found in 80 small squares by 80, thus determining the average number per small square or in 0.00025 c.mm.

16. Multiply the average number of cells per 0.00025 c.mm. by 4000 to determine the number of cells in 1 c.mm. of diluted blood, and by 200 to determine the number in 1 c.mm. of undiluted blood.

17. *Therefore in routine work count the number of cells in 80 small squares and add four ciphers.*

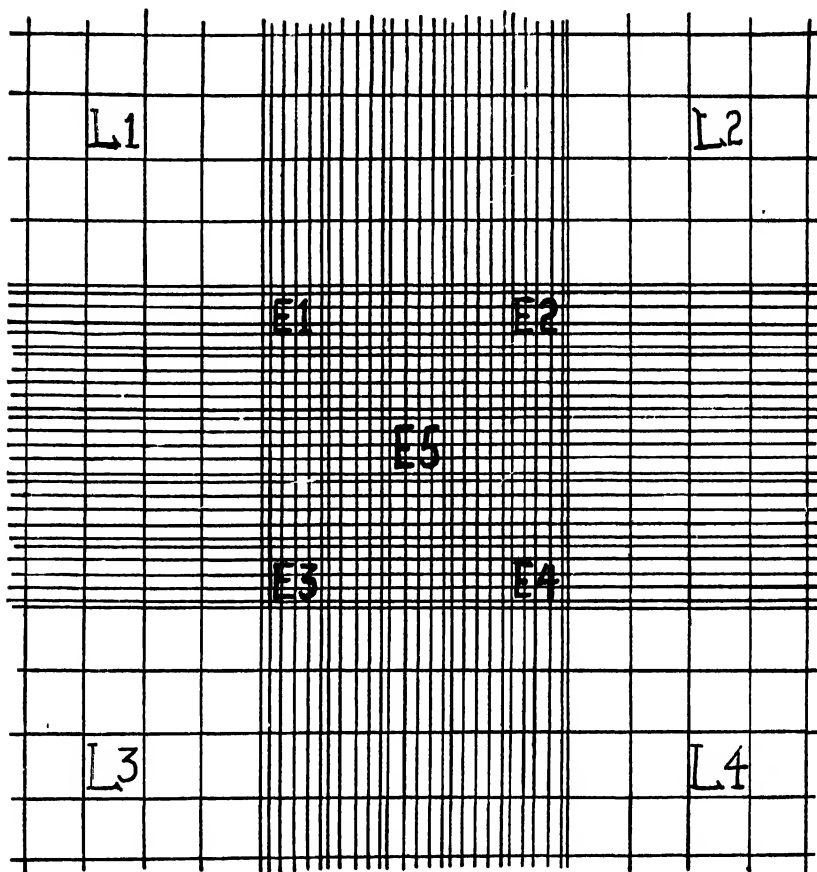


FIG. 46.—COUNTING OF ERYTHROCYTES AND LEUKOCYTES (IMPROVED NEUBAUER CHAMBER)

The numbers  $E_1$ ,  $E_2$ ,  $E_3$ ,  $E_4$  and  $E_5$  indicate five large squares (80 small squares) for counting erythrocytes;  $L_1$ ,  $L_2$ ,  $L_3$  and  $L_4$  indicate the parts of the slide used in counting leukocytes. (From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

**Sources of Error.**—The following sources of error must be kept in mind and carefully avoided: (a) Inaccurate dilution due to faulty pipets or technic; (b) too slow manipulation, allowing a little of the blood to coagulate; (c) inaccuracy in the counting chamber and especially in its depth due to inaccurate coverglass, faulty manufacture, loosening of parts, etc.; (d) presence of yeasts and other artefacts in the diluting fluid; (e) delay in filling counting chamber after shaking pipets; (f) uneven distribution of the cells.

### METHODS FOR DETERMINING THE VOLUME OF PACKED ERYTHROCYTES

**Principles.**—1. The determination of the volume of packed erythrocytes in a given amount of blood is a valuable procedure in the diagnosis and differential diagnosis of the anemias. It is of aid in determining the type and degree of anemia. In



conjunction with an erythrocyte count changes in the size of erythrocytes may be detected. Consequently, the examination should be made in all cases of anemia.

2. Various hematocrits have been employed for the determination. Those of Wintrobe and Haden (employing the Sanford-Magath tube) also furnish information of value on alterations in the quantities of leukocytes and platelets as well as the color and opacity of the blood plasma.

3. The volume of packed erythrocytes in 100 cc. of blood is normally as follows:

Infants .....	49 to 60 cc.
Children .....	44 to 52 cc.
Men .....	39 to 52 cc.
Women .....	35 to 48 cc.



FIG. 47.—VOLUME OF PACKED ERYTHROCYTES (WINTROBE)

A, normal blood; B, simple chronic anemia (normocytic); C, pernicious anemia (macrocytic); D, chlorosis (microcytic); E, erythremia (normocytic); F, chronic myelocytic leukemia. (From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co. New York.)

4. From the erythrocyte count, hemoglobin estimation and cell volume, the mean corpuscular hemoglobin, mean corpuscular volume and other indices may be determined.

**Wintrobe Method.**—1. The hematocrit is a flat-bottomed narrow glass tube 11 cm. in length, of about 2.5 to 3 mm. inside diameter (Fig. 47). A centimeter-millimeter scale, commencing at the level of the inside bottom, is etched on the glass.

2. Prepare a solution containing 0.8 gm. of potassium oxalate and 1.2 gm. of ammonium oxalate in 100 cc. of distilled water. Place exactly 0.5 cc. in test tubes, evaporate off the water and thoroughly dry before using. This mixture of oxalates does not change the volume of erythrocytes, so that no correction of the final reading is necessary.

3. Place 5 cc. of venous blood in one of these oxalate test tubes. Mix gently, but thoroughly.

4. Fill the Wintrobe hematocrit tube exactly to the 10 mark at the right side of the scale by means of a pipet made from glass tubing drawn out to such length that it can be passed to the bottom of the tube. Avoid forming bubbles of air.

5. Close the tube by means of a rubber stopper to prevent evaporation and centrifuge long enough for complete packing of the cells, usually for 30 minutes at about 3000 revolutions per minute.

6. The level of the packed erythrocytes multiplied by 10 gives the volume per 100 cc. of blood. The cell volume in per cent may be determined by dividing the height of the column of packed erythrocytes by the total height of the column of cells and plasma and multiplying by 100.

7. Above the deep red layer of packed erythrocytes is a reddish-gray layer of packed leukocytes and platelets. In normal blood this varies from 0.5 to 1 mm. in thickness, each 0.1 mm. corresponding approximately to 1000 leukocytes per c.mm. The thickness of this layer depends on the number of leukocytes, the kind of leukocytes, and the quantity of platelets. At the top of the erythrocyte column, just under the layer of leukocytes and platelets, a black line may be observed. According to Baumberger,<sup>4</sup> this represents a layer of erythrocytes in which the oxyhemoglobin has been reduced to hemoglobin by the metabolic activity of the leukocytes.

8. The color and opacity of the plasma should be observed and recorded.

9. After use, the tube should be thoroughly washed with water followed by alcohol and ether, and thoroughly dried. From time to time it may need to be cleaned with antiformin to remove a thin film of coagulated protein which may accumulate on the glass.

**Haden Method (Modified).**—1. In a Sanford-Magath hematocrit tube (Fig. 48) place 1 cc. of a 1.1 per cent solution of sodium oxalate in distilled water.

2. Add exactly 5 cc. of venous blood at the bedside, that is, up to the 6 cc. mark. Mix by inverting once or twice.

3. Centrifuge for 20 minutes at about 3000 revolutions per minute or until a maximal packing of the cells has occurred.



FIG. 48.—THE  
SANFORD-MAGATH  
HEMATOCRIT  
TUBE

4. With normal blood the reading is about 2.4 cc. Multiply by 20 to obtain the red corpuscle volume per 100 cc. of blood.

5. If less than 5 cc. of blood has been used, divide the reading of packed cells by the number of cubic centimeters of blood used and multiply by 100, as per the following example:

$$\frac{1.6 \text{ cc. packed erythrocytes}}{4.0 \text{ cc. blood used}} = 0.4 \times 100 = 40 \text{ (red cell volume per 100 cc.)}$$

**Van Allen Method.**— This method has the advantage of using capillary blood and is especially useful in the case of children.



FIG. 49



FIG. 50

FIG. 49.—THE VAN ALLEN  
HEMATOCRIT TUBE

FIG. 50.—SPRING SEALING CLIP

1. Prick the finger and discard the first drop or two of blood. Collect a large drop.

2. Accurately draw blood up in a Van Allen hematocrit tube (Fig. 49) to the mark 10.

3. Draw the blood up the stem a short distance and then draw the diluting fluid (1.6 per cent solution of sodium oxalate in distilled water) into the bulb until it is about half full.

4. Close the tube by means of a broad strong rubber band or the special spring sealing tip (Fig. 50) and centrifuge (shaft end down) at about 2500 r.p.m. or more, for 15 to 30 minutes.

5. The height of the column of packed erythrocytes represents the relative volume of the blood made up by these cells. No correction for dilution is necessary. The stem of the tube is divided into 10 units of 10 divisions each. Each division represents 1 per cent.

## METHOD FOR DETERMINING THE MEAN CORPUSCULAR VOLUME

**Principle.**—The mean or average size of the individual erythrocyte in cubic microns may be calculated from the volume and the numbers of erythrocytes in a given quantity of blood. *It is recommended for routine use in the place of the volume index,* as it does not involve the use of any normal figures in its calculation and has the same clinical value. If the cells are larger than normal, the condition is called *macrocytosis*, and if associated with anemia, *macrocytic anemia*. If smaller than normal, the condition is called *microcytosis* and if associated with anemia, *microcytic anemia*. If the cells are normal in size, then it is called *normocytic*, and if associated with anemia, *normocytic anemia*.

**Procedure.**—1. Determine the volume of packed cells per 100 cc. of blood.

2. Make an erythrocyte count.

3. Divide the number of cc. of packed cells per 100 cc. of blood by the number of millions of erythrocytes per c.mm. and multiply by 10.

$$\frac{\text{Volume of packed cells per 100 cc. of blood}}{\text{Erythrocytes in millions per cubic millimeter}} \times 10 = \text{Mean corpuscular volume in cubic microns.}$$

Example: Volume of packed cells = 31 cc.

Erythrocyte count = 2,400,000.

$$\frac{31}{3.4} \times 10 = 91 \text{ cubic microns (M.C.V.).}$$

4. The normal values: 105 at birth falling rapidly to childhood level 87 to 73; adults 82 to 92.

### METHOD FOR DETERMINING THE MEAN CORPUSCULAR HEMOGLOBIN

**Principle.**—The mean or average amount of hemoglobin by weight per cell is calculated and expressed in micromicrograms. *It is recommended for routine use in the place of the color index* as it does not involve the use of any normal figures in its calculation and has the same clinical value.

If the amount of hemoglobin per cell is larger than normal, it is *hyperchromic* and if associated with anemia, *hyperchromic anemia*. If the amount is smaller than normal it is *hypochromic* and if associated with anemia, *hypochromic anemia*. If the amount is within the normal range then it is *normochromic* and if associated with anemia, *normochromic anemia*.

**Procedure.**—1. Determine the hemoglobin in grams per 100 cc. of blood.

2. Make a total erythrocyte count.

3. Divide the number of grams per 100 cc. of blood by the number of millions of erythrocytes per c.mm. and multiply by 10.

$$\frac{\text{Hemoglobin in grams per 100 cc.}}{\text{Erythrocytes in millions per c.mm.}} \times 10 = \text{Mean corpuscular hemoglobin in micromicrograms.}$$

Example: 12.0 gms. hemoglobin per 100 cc. of blood.

4,200,000 erythrocytes per c.mm.

$$\frac{12.0}{4.2} \times 10 = 28.6 \text{ micromicrograms (M.C.H.)}$$

4. The normal values: Infants 40 at birth falling rapidly to childhood level 33 to 27; adults 29 to 31.

### METHOD FOR DETERMINING THE MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION

**Principle.**—This is the mean or average hemoglobin concentration in gm. per 100 cc. of packed erythrocytes. It is calculated by dividing the hemoglobin in grams per 100 cc. of blood by the volume of packed erythrocytes in cc. per 100 cc. of blood and multiplying by 100. *It has the same clinical value as the saturation index and is recommended as a substitute for it.*

**Procedure.**—1. Determine the hemoglobin in grams per 100 cc. of blood.

2. Determine the volume of packed cells in cc. per 100 cc. of blood.

3. Divide the grams of hemoglobin per 100 cc. of blood by the number of cc. of packed cells per 100 cc. of blood and multiply by 100.

$$\frac{\text{Hemoglobin in grams per 100 cc. of blood}}{\text{Volume of packed cells in cc. per 100 cc. of blood}} \times 100 = \text{Mean corpuscular hemoglobin concentration.}$$

Example: 15.6 gms. hemoglobin per 100 cc.  
45 cc. packed erythrocytes per 100 cc.

$$\frac{15.6}{45} \times 100 = 35 \text{ gms. per 100 cc. of packed cells.}$$

(M.C.H.C.)

4. The normal range: Infants about 45 at birth falling rapidly to childhood level 41 to 34; adults 33 to 37.

### METHODS FOR DETERMINING THE VOLUME INDEX, COLOR INDEX AND SATURATION INDEX

1. These indices express the following ratios:

$$\text{Volume index} = \frac{\text{Mean corpuscular volume—patient}}{\text{Mean corpuscular volume—normal}}$$

$$\text{Color index} = \frac{\text{Per cent of normal hemoglobin concentration}}{\text{Per cent of normal erythrocyte count}}$$

$$\text{Saturation index} = \frac{\text{Mean corpuscular hemoglobin concentration—patient}}{\text{Mean corpuscular hemoglobin concentration—normal}}$$

2. Each of these ratios involves accepting normal values for comparison with the findings in the patient. Different normal values have been used by different workers. Sometimes normal values for the same age and sex have been employed. Sometimes a single normal value has been taken and used for both sexes at any age. Sometimes values have been taken in round numbers to facilitate calculation. *It follows that no interpretation can be given to these indices unless the author has defined his method of calculating the index.* The normal variability can be taken as about 8 per cent.

3. Since the calculation of the values of the mean corpuscular volume, hemoglobin and hemoglobin concentration do not require the use of normal figures and have the same clinical value, it is recommended that the *mean corpuscular volume be used in place of volume index* (see page 64); *the mean corpuscular hemoglobin in the place of color index* (see page 65); and *the mean corpuscular hemoglobin concentration* (see page 65), *in place of the saturation index.*

### METHOD FOR COUNTING TOTAL LEUKOCYTES

**Principles.**—1. Blood is accurately diluted 1:20 with a fluid producing complete hemolysis of erythrocytes, but without injury to the leukocytes. The corpuscles contained in 0.4 c.mm. of the diluted blood are then counted in a special chamber and this number multiplied by 50 to obtain the number in 1 c.mm. of undiluted blood. This constitutes the usual method.

2. The normal number of leukocytes is usually stated to be from 5000 to 10,000

per c.mm.; however, normal counts may be slightly lower or higher according to age, influence of digestion, *time of day*, and other little understood factors. There is a greater normal fluctuation in the number of leukocytes than in the erythrocytes. In the morning the number is usually the lowest and gradually rises until evening, probably as a result of exercise. There may be as much as a 100 per cent increase, but still the count may fall within the normal range. It is therefore advisable to *record the time of day a leukocyte count is made*. A count made during the afternoon may be 2000 higher than one made in the morning. Such an increase may not be necessarily due to disease.

**Procedure.**—1. Draw blood to the mark 0.5 of the Thoma pipet marked 11 or fill the stem of the Trenner pipet, as described in the method for counting erythrocytes.

2. Draw up diluting fluid to mark 11, thus making a dilution of 1:20.

#### DILUTING FLUID FOR LEUKOCYTES

Acetic acid .....	3 cc.
Water .....	97 cc.

Add a few drops of an aqueous solution of methylene blue to slightly color the fluid. Filter frequently to remove yeasts, molds and artefacts. N/10 hydrochloric acid can also be used as a diluent and after the leukocyte count has been made the remaining fluid can be used for determining the hemoglobin by the Haden method.

3. Rotate the pipet well for several minutes, holding it in a horizontal position: finally shake sideways.

4. Blow out several drops.

5. Fill the counting chamber in exactly the same manner as described for the counting of erythrocytes.

6. Allow about 3 minutes for the cells to settle.

7. Center the light and focus exactly as described for the erythrocyte count.

8. Examine with a low power objective to see that the cells are evenly distributed. Count with the 16-mm. objective and a medium ocular which will include a square millimeter in the field.

9. If the improved Neubauer counting chamber is used, count the cells in the four corner blocks,  $L_1$ ,  $L_2$ ,  $L_3$ , and  $L_4$  shown in Figure 46. East of these 4 square millimeter areas is subdivided into 16 squares to facilitate counting. In counting the cells include those touching the inner lines on the right and top, but omit those touching the lines on the left and bottom. The difference between the largest and smallest number of leukocytes in any two blocks should not exceed 10. To obtain the total leukocytes per c.mm. of undiluted blood, multiply the total by 50, or divide the count by 2, and add 2 ciphers. This should be repeated upon a second and a third slide and the average taken.

10. If some other rulings are used, count the leukocytes in several square millimeter areas. Find the average, multiply by 10 to find the number in 1 c.mm. of diluted blood, and by 20 (the dilution). Or, multiply the average by 200.

11. In cases of *very low leukocyte counts* use the Thoma pipet. Draw blood to the 1 mark and diluting fluid to the 11 mark, which gives a 1:10 dilution. Count the cells in four sq.mm. areas ( $L_1$ ,  $L_2$ ,  $L_3$  and  $L_4$  of Fig. 46) and multiply by 100 to give the total per c.mm. of undiluted blood. Since such counts are only approxi-

mately accurate, it is advisable to use the Levy counting chamber with the Fuchs-Rosenthal ruling as employed for counting leukocytes in spinal fluid. It is divided into 16 large squares of 0.2 c.mm. each with the coverglass adjusted. Count the leukocytes in 5 of the squares and multiply by 10 (the dilution factor).

12. In cases of *very high leukocyte counts* (40,000 or above) it is advisable to use a red corpuscle pipet. Draw blood to the 1 mark and the acetic acid diluting fluid to the 101 mark, which gives a 1:100 dilution. Count the leukocytes in 4 squares ( $L_1$ ,  $L_2$ ,  $L_3$  and  $L_4$  of Fig. 46) divide by 4 and multiply by 1000. If the count is as high as 100,000 or more, use the red corpuscle pipet with blood drawn to the 0.5 mark and the acetic acid diluting fluid to the 101 mark, which gives a 1:200 dilution. Count the leukocytes in 4 squares ( $L_1$ ,  $L_2$ ,  $L_3$  and  $L_4$  of Figure 46), divide by 4 and multiply by 2000.

**Sources of Error.**—With experience and care the range of error in counting leukocytes is easily kept within 5 per cent. Sources of error include the following: (1) Inaccurate dilution; (2) too slow manipulation allowing coagulation in the pipet; (3) improper shaking of the pipet; (4) failure to expel several drops before filling the counting chamber; (5) inaccuracy in the depth of the counting chamber; (6) uneven distribution of the cells; (7) overflowing of the counting chamber; (8) bubbles in the counting chamber; (9) touching the coverglass with the objective and (10) the presence of yeasts and artefacts in the diluting fluid mistaken for leukocytes.

Since the nuclei of erythroblasts are not dissolved by the acetic acid of the diluting fluid, another source of error is (11) *mistaking nuclei of red blood corpuscles for leukocytes*. This is especially important in erythroblastosis fetalis and Cooley's anemia in which large numbers of erythroblasts occur. Consequently, whenever stained blood films show the presence of large numbers of these cells, it is necessary to correct the total leukocyte count as per the following example:

$$\begin{array}{l}
 \text{Uncorrected total leukocyte count: } 35,500 \text{ c.mm. (including} \\
 \quad \text{leukocytes and nuclei of erythrocytes)} \\
 \text{110 erythroblasts per 100 leukocytes in the differential count} \\
 \quad \text{of a stained blood film} \\
 35,500: X :: 210 : 100 \\
 210X = 3,550,000 \\
 \frac{3,550,000}{210} = 16,904 \text{ corrected total leukocyte count.}
 \end{array}$$

## METHODS FOR PREPARING AND STAINING BLOOD FILMS

**Principles.**—1. An examination of the erythrocytes and different kinds of leukocytes possesses a great deal of diagnostic value. For this purpose stained preparations are superior to unstained or wet preparations.

2. The blood must be prepared in thin, evenly distributed smears on slides or coverglasses. *Well prepared films or smears are absolutely essential for accurate results.* For routine work a variety of staining methods are available. Preference should be given the polychrome stains, that is, those capable of staining the neutral, acid and basic granules of leukocytes. Poorly prepared and faultily stained smears cannot give results of acceptable accuracy.

**Procedure.**—1. Perfectly clean grease-free slides are recommended. *New slides*

should be washed in soap and water, rinsed with tap water, placed in 95 per cent alcohol for 15 minutes and dried with a piece of lint-free towel. *Used slides* may be prepared by removing the immersion oil with xylol followed by boiling in a very weak soap powder solution, thoroughly rinsing in tap water, immersing in a 1:1000 solution of nitric acid overnight, thoroughly rinsing in running tap water, and drying with a piece of lint-free towel.

2. Cleanse a finger or lobe of the ear and prick as previously described for the collection of capillary blood.

3. Touch the end of a slide to a large drop of blood; then spread the drop with a second slide. As soon as the blood has spread entirely across the end of the spreader slide (held at an angle of about 45 degrees) with a rather quick movement *push* (do not pull) the spreader toward the other end of the under slide (Fig. 51). The film should be neither too thin nor too thick (Fig. 52). Allow the blood to dry.

4. Blood smears may also be made on coverglasses. They should be perfectly clean and free of grease. Take up a small drop of blood on one without touching the surface of the skin and place it on the second in such manner that the corners do not overlap. As soon as the blood spreads out between the glasses, draw them apart in a plane parallel to their surface. Dry in the air. This method is preferred by some clinical pathologists.

5. A combination of the slide and coverglass methods is that of Beacom in which smears are prepared as follows: A small drop of blood is placed near the end of a clean slide and a coverglass is dropped on it; if the blood does not flow out properly, very gentle pressure may be used. The slide is then held on a table with the left hand and the first 2 fingers of the right are placed near the edges close to the left end of the coverglass. Then, with an even, fairly rapid pull, it is slid along the slide in the direction of its long axis with only enough pressure to keep the fingers from slipping from the spreader. This leaves a smooth, even smear on the slide for examination.

**Wright's Method.**—This method is recommended for routine purposes and is commonly employed.

1. A simple and satisfactory method for preparing the stain consists in placing 1.0 gram of the dry powder (National Aniline and Chemical Company of New York) in a clean, dry dark brown bottle and adding 250 cc. of C.P. acetone-free methyl alcohol. Stopper tightly, keep at room temperature in a dark place and shake daily for 5 minutes over a period of a week, when it is ready for use. As required, small amounts of this stock stain may be filtered into a small bottle. The stain will keep for over 6 months.

2. It is advisable to use a buffer solution (pH 6.4) in staining, prepared by dissolving 6.63 gm. of monobasic potassium phosphate and 3.2 gm. of dibasic sodium phosphate in 1000 cc. of distilled water.

3. Without previous fixation, cover the film with a counted number of drops of the stain. There must be plenty of stain in order to avoid too great evaporation and consequent precipitation. When slides are used, the stain may be confined to the desired area by 2 heavy wax-pencil marks.

4. After 1 minute add the same number of drops of buffer solution with a second dropper.

5. Allow the mixture to remain 4 minutes. A longer period of staining may produce



a precipitate. Eosinophilic granules are brought out best by a shorter period of staining.

6. Do not pour off the stain but flood the preparation with tap water and wash for 30 seconds.

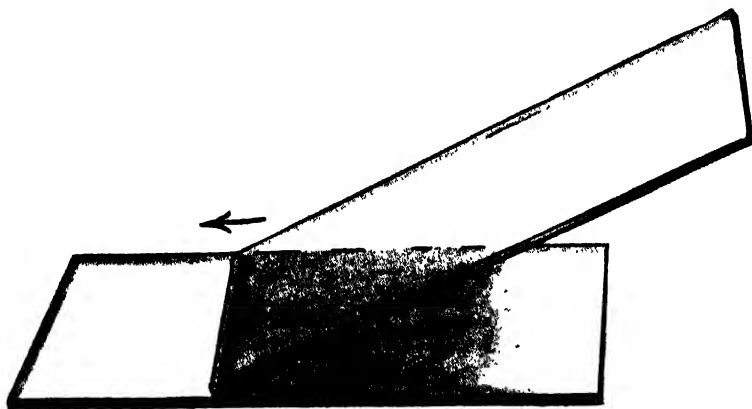


FIG. 51.—METHOD OF PREPARING A BLOOD SMEAR

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

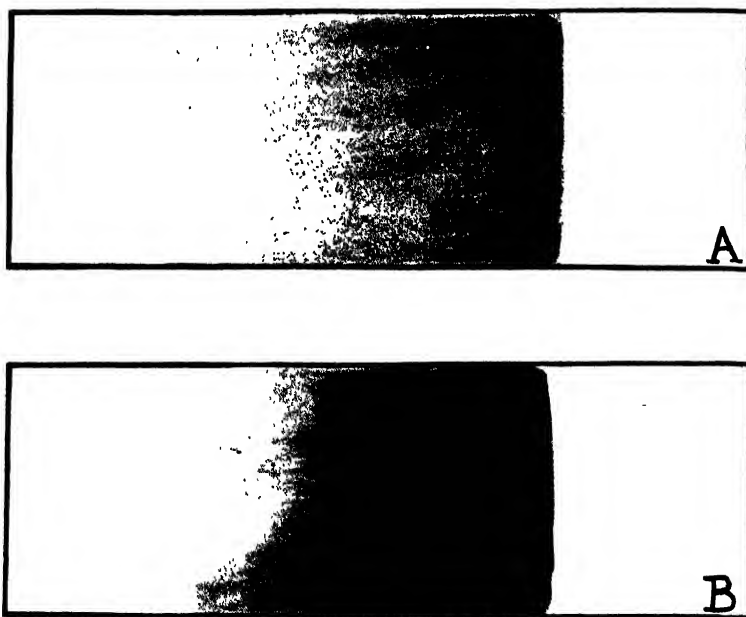


FIG. 52.—BLOOD SMEARS

*A*, a satisfactory thin, evenly spread smear; *B*, an unsatisfactory thick smear. (From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

7. Dry best by waving high above a Bunsen flame. Films on slides may be examined directly with the oil-immersion objective. Films on coverglasses should be mounted in balsam.

**Giemsa Method.**—This stain is probably the best modification of the Romanowsky stain for malarial and other blood parasites and is also very satisfactory as a routine blood stain.

1. The stain may be prepared as follows: Place 75 cc. of C.P. acetone-free methyl alcohol and 25 cc. of acid-free glycerin in a beaker. Put in a water bath and add 0.75 gms. of imported dry powder Giemsa stain (Akatos, Inc., 114 Liberty Street, New York City) and warm to 60° C. Stir with a glass rod. Filter through No. 4 dry filter paper into a dry clean bottle and keep well stoppered. For use, dilute 1 part of stock stain with 4 parts C.P. acetone-free methyl alcohol every 2 weeks.

2. Cover film with 15 drops and stain for 1 minute.

3. Add 30 drops of distilled water, mix well and stain for 5 minutes.

4. Wash by flooding with distilled water.

5. Blot dry or stand on end and dry in air.

**Combination of Wright's and Giemsa's Methods.**—This method is especially useful in the leukemias for bringing out basophilia more clearly and in finer degrees. Three types of staining occur: (a) Slight basophilia in which the cytoplasm is almost colorless or pinkish; (b) moderate basophilia in which the cytoplasm is a sky blue and (c) marked basophilia in which the cytoplasm is a dark blue. With this combined staining, differentiation between young and old lymphocytes and young and old monocytes is more accurately made, and the azure granules are more clearly defined.

1. Stain as in the Wright method.

2. Pour off the stain and immediately cover with a mixture of 3 parts Giemsa stain and 2 parts of triple distilled water for 4 minutes.

3. Wash briefly and dry.

**Jenner's Method.**—This method stains nuclei poorly and is much inferior to the Wright's stain for the malarial parasite since it does not give the so-called "Romanowsky staining."

1. The stain, which may be purchased in the form of tablets or as a powder, is prepared by dissolving 0.5 gm. in 100 cc. of C.P. acetone-free methyl alcohol.

2. Cover the film with the stain for 3 to 5 minutes.

3. Wash with tap water until a lavender color is obtained.

4. Blot and dry in the air.

**Pappenheim's Method.**—This pyronine-methyl green stain is very satisfactory for the study of erythrocytes and lymphocytes. Polychromatophilia is well demonstrated, the affected corpuscles staining more or less red. All nuclei are blue to reddish-purple; basophilic granules and cytoplasm of lymphocytes are stained red.

1. The stain is prepared by mixing 30 to 40 cc. of a saturated aqueous solution of methyl green with 10 to 15 cc. of a saturated aqueous solution of pyronine. The mixture will keep for about a month. If it is found that one of the dyes stains too deeply, it may be reduced by adding more of the other dye.

2. Fix the film with heat.

3. Cover with stain and allow to remain for ½ to 5 minutes.

4. Wash thoroughly with distilled water and allow to dry.

**Washburn's Peroxidase Method.**—This method is sometimes employed as an aid in the identification of myelocytes in the differential diagnosis of the leukemias. It is based upon the principle that a study of the oxidizing ferments of leukocytes aids in differentiating those of myeloid origin from those of other origin.

touching the skin. Place on a slide; do not put pressure on the cover-slip. Rim the cover-slip with *thick* immersion oil or vaseline to exclude oxygen and prevent evaporation.

6. Place slide in an electric incubator or a microscopic stage (Chicago Surgical and Electrical Company) at a temperature of 100.4° F. ( $\pm 1^\circ$ ).

7. Examine at intervals over a period of at least 20 to 40 minutes, which is satisfactory for the staining of vacuoles and granules and permits comparisons between granulocytes and endothelial leukocytes. Normal granulocytes do not begin to show vacuoles until after 30 to 40 minutes, while the vacuoles of lymphocytes and endothelial leukocytes are usually stained in 10 to 15 minutes. Mitochondria are usually stained in 5 to 10 minutes. Janus green is quite toxic. Preparations which have stood 1 to 2 hours may be quite satisfactory for differential counts but not for qualitative analysis of the functional state of the cells.

8. The characteristics of cells of normal blood with supravital staining have been summarized by Beck <sup>6</sup> as shown in Table 5 on page 76.

### CLASSIFICATION OF NORMAL AND ABNORMAL LEUKOCYTES

The normal and abnormal leukocytes of the blood are classified according to their size, morphology and staining reactions, as summarized in Table 6. Those occurring normally are divided into 3 kinds, namely, (a) *lymphocytes* derived from the lymphoid tissues; (b) *monocytes* or endothelial leukocytes, derived from the reticulo-endothelial system and (c) *granulocytes*, derived from the bone marrow. Their numbers per c.mm. of blood vary under normal conditions according to age, as shown in Table 3. The granulocytes are so-called because of the presence of granules in their cytoplasm and are subdivided, according to their staining reactions, into *neutrophils*, *eosinophils* and *basophils* (Fig. 53).

*Lymphocytes* (Plate I) vary in size from about that of an erythrocyte to that of a neutrophil (7 to 15 microns). The nucleus is round and stains deeply with the basic stain. The smaller ones stain more deeply and have a small amount of cytoplasm. The larger ones often stain less intensely and have more cytoplasm and in some may be seen several round, reddish-purple, azurophilic granules. Occasionally, forms with indented nucleus appear. It is generally believed that the large, less deeply staining forms are the younger types which become smaller upon reaching maturity. An increase of these cells is called *lymphocytosis* and a decrease *lymphopenia*.

*Monocytes* (Plate I) include cells which were formerly called large mononuclear leukocytes and transitionals. They are also known as endothelial leukocytes or endotheliocytes. The nucleus is less deeply stained than that of the lymphocytes, is usually indented, and at times is horseshoe shaped. The chromatin material in the nucleus has a skein-like appearance. Those with round nuclei are often difficult to distinguish from lymphocytes. There is a wider band of cytoplasm than in the lymphocytes. The lymphocytes are usually not as large as neutrophils, while the monocytes are usually larger. The chromatin of lymphocytes is more granular in appearance. An increase is called *monocytosis*.

*Neutrophils* (Plate I) are easily recognized by an irregular-shaped and lobulated nucleus, for which reason they are commonly known as "polymorphonuclears". The nucleus may be ribbon, band-like or segmented. The segments vary in number from

# PLATE I

Lymphocytes



Monocytes



Nonfilament  
neutrophils



Filament neutrophils



Eosinophils



Basophils



Myeloblasts



Promyelocytes



Myelocytes  
(Neutrophilic, eosino-  
philic and basophilic)



Türk cells



Plasma cell



Degenerated nucleus  
("Basket-cell")



NORMAL AND ABNORMAL LEUKOCYTES (WRIGHT'S STAIN)



1 to 6 or 7 and are all connected by narrow nuclear bands. The cytoplasm contains numerous fine granules which do not stain definitely either blue (basic) or red (acid) and hence are regarded as neutral or neutrophilic. They may undergo an increase, designated as "*neutrophilia*", or a decrease, called "*neutropenia*". They have been

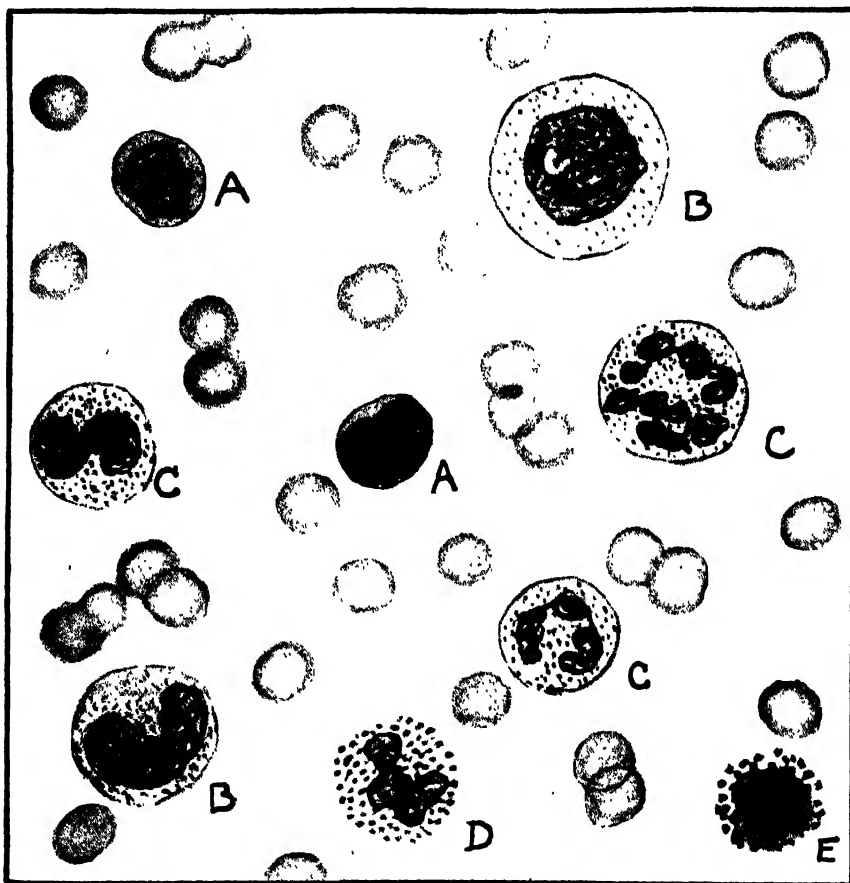


FIG. 53.—NORMAL LEUKOCYTES OF THE BLOOD (A COMPOSITE DRAWING)

A, lymphocytes; B, monocytes; C, polymorphonuclear neutrophils; D, eosinophil; E, basophil.

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

subdivided by Arneth, Schilling, and others according to the number and shapes of nuclear segments.

*Eosinophils* (Plate I) are granulocytes similar to the neutrophils except for a difference in the size and staining properties of the granules, which are round or oval and large enough to be distinctly outlined. They stain pink to bright red (acid stain) with Wright's stain. An increase is called "*eosinophilia*" and a decrease "*eosinopenia*".

*Basophils* (Plate I) are granulocytes similar to the neutrophils except that they contain granules which are larger and stain deep purple (basic stain) with Wright's stain. The nucleus is usually without distinct lobulation. The cell itself is slightly

**TABLE 5.—CHARACTERISTICS OF CELLS OF NORMAL BLOOD WITH SUPRAVITAL STAINING**

Cell	Granules	Vacuoles	Mitochondria	Motility	Nucleus
Segmented neutrophil	Very fine, yellowish, pink or orange, light and dark. Some are refractive and uncolored.	Not in normal cell. In acute infections after 15 minutes. In any preparation after 30 minutes. Very few, small and red.	Few or lacking. If present, very fine short rods scattered throughout cytoplasm.	Ameboid. Fairly rapid. Streaming of cytoplasm and granules.	Separated into lobes.
Eosinophil	Large, slightly oval, golden yellow. Uniform in size. At end of 1 or 2 hours some may be reddish brown.	Develop more slowly than in neutrophil. After some time may be 2 or 3 bright red.	Rarely seen. Few very fine on periphery or scattered.	Ameboid. Very active. Streaming of cytoplasm and granules.	Two lobes connected by bridge.
Basophil	Round, vary slightly in size. Larger than neutrophil, smaller than eosinophil. Brick red. Those out of focus look pink.	Rarely seen. If present develop slowly, quite large and red.	Rarely found. May be like in neutrophil.	Ameboid. Sluggish. Less than eosinophil and neutrophil.	Less indented than eosinophil.
Nonmotile neutrophil	Large, highly refractive, do not stain.	None.	Very few, fine or lacking.	None.	Swollen. Nuclear bridges cannot be seen.
Neutrophilic metamyelocyte	Cytoplasm filled, dark pink or golden brown. Slightly larger and stain more sharply than neutrophil.	Not in normal cell.	Few arranged around periphery.	Seldom motile. May be very slight ameboid in some.	Oval or indented.

Lymphocyte	None.	They stain in 15 minutes. At 1 hour only 2 or 3. red, small and tend to clump.	Adherent to nucleus or <i>clumped in one area</i> ; one or both. Medium sized rods, larger than in other mature cells.	Very slightly motile.	Round, oval or indented.
Monocyte (Endothelial leukocyte)	None.	Always present. Stain in 10 to 15 minutes. Salmon brown. Change to red on standing. Rosette in hof of nucleus or scattered throughout. All sizes, some very large.	Numerous. Usually at periphery or mixed with vacuoles at hof of nucleus. Same size as those of neutrophil.	Less than in granular cells. Do not move by pseudopodia. There is gradual change of entire cytoplasm. A surface film motility.	Oval or indented.



smaller than the neutrophil. They are also called "mast cells". An increase is called "*basophilia*" or "*basophilic leukocytosis*".

**Shifts to the Left and Right.**—In 1904 Arneth attempted to measure the relative age of the circulating neutrophilic leukocytes from the morphology of the nucleus. He showed that the nucleus of the neutrophil, in its development from the myeloblast in the marrow, becomes gradually more indented and divides with age into an increasing number of separate lobes or segments. The shape of the nucleus is thus an index of the maturity of the cell (Fig. 54). He divided the neutrophils into 5 major groups

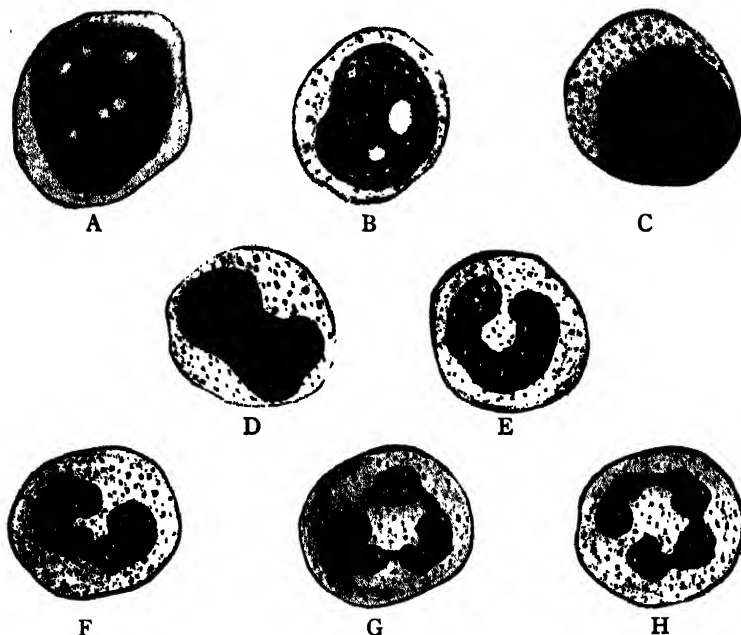


FIG. 54.—IMMATURE ("SHIFT TO THE LEFT") AND MATURE ("SHIFT TO THE RIGHT") NEUTROPHILS





















A, myeloblast; B, promyelocyte; C, myelocyte; D and E, nonfilamented or immature neutrophils (metamyelocytes); F, G and H, filamented or mature neutrophils.

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

and numerous subgroups on the basis of nuclear configuration (Fig. 55). The nucleus in group I has only 1 lobe and in group V it has 5 or more lobes. Arneth suggested the terms "*shift to the left*" to indicate an increase in young cells or in those with fewer lobes, and "*shift to the right*" to designate an increase in older cells or in those with a larger number of lobes. Arneth's complete classification is unfortunately entirely too complicated for practical laboratory application. His observations on the relation of the nuclear form to the age of cell, however, have been accepted as fundamentally correct, and have stimulated further study and classification of the maturity of the neutrophils. Such studies have proved of great value in clinical hematology.

Schilling, in 1911, suggested a classification of the neutrophilic leukocytes (Fig. 56) which has been widely employed, especially in the study of diseases related to infection. He divides the neutrophils in the order of their age into 4 groups: (1) Myelocyte;

(2) juvenile nuclear in which the nucleus has become indented; (3) stab or staff  
nuclears in which the nucleus is T-, V- or U-shaped without division into segments;  
and (4) segmented nuclears which are fully differentiated neutrophils with distinct

CLASS					
I					
One Nucleus	M 0.0%	W 0.2%	T 5.0%		
II					
Two-Lobed Nuclei	2K 0.27%	2S 23.5%	1K 1S 11.7%		
III					
Three-Lobed Nuclei	3K 23%	3S 56%	2K 1S 16.7%	1K 2S 16.4%	
IV					
Four-Lobed Nuclei	4K 3.8%	4S 0.07%	3K 1S 6.4%	3S 1K 1.6%	2K 2S 4.7%
V					
Five-Lobed Nuclei	5K 1.0%	4K 1S 0.4%	3K 2S 0.4%	4K 2S 0.07%	3K 3S 0.07%

M=Myelocyte W=Slightly Indented Nucleus  
T=Deeply Indented Nucleus  
K=Round Piece S=Bent Piece

FIG. 55.—ARNETH'S CLASSIFICATION OF THE NEUTROPHILS (HADEN)

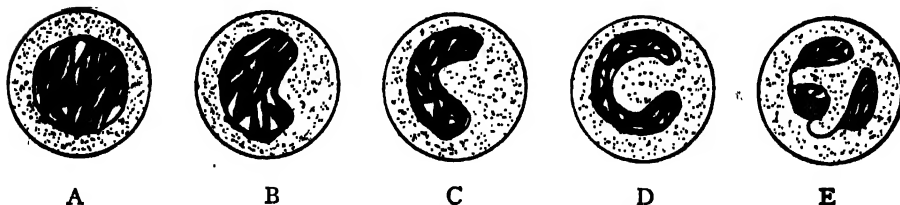


FIG. 56.—SCHILLING'S CLASSIFICATION OF THE NEUTROPHILS

A, myelocyte (normal 0); B, juvenile neutrophil (normal 0.1 per cent); C and D, stab  
neutrophils (normal 3 to 5 per cent); E, segmented neutrophils (normal 51 to 67 per cent).

segmentation into from 2, 5 or more lobes. Schilling's classification is much simpler  
than Arneth's and recognizes 2 types of "shift to the left". Arneth, in his "shift to the  
left", considered that the increased number of cells with fewer lobes was caused only  
by the rapid outpouring of leukocytes from the marrow in response to an acute need

before growth and differentiation were complete. Schilling designates this a *regenerative* "shift to the left". It is associated with a high total neutrophil count. In other instances a depression of bone marrow function seems to prevent the complete differentiation of the neutrophils, so that they develop only to a certain point and emerge into the circulation at this stage. The differential count in such cases shows an increased proportion of immature forms due to the depressed marrow function but the total neutrophil count is low. Schilling designates this type of reaction as a *degenerative* "shift to the left". Other evidences of degeneration of the neutrophils are also seen in these, such as loss of structure and narrowing and deep staining of the nucleus,

irregularity in size and staining reaction of the granules in the cytoplasm and the appearance of vacuoles.

Schilling, in classifying the neutrophils, rightly places the emphasis on the more immature forms. Arneth has emphasized the subdivision of the segmented or more mature types. It is also often difficult to determine when the nucleus of any given cell has become segmented. Cooke and Ponder suggest a simple criterion, which is now known by their names, for determining division of the nucleus. The nucleus never divides completely. The lobes are connected either by a fine filament or by denser bridges of nuclear material. Cooke and Ponder do not classify a nucleus as divided if the segments are connected by other than a fine chromatin thread (Fig. 57). By using this criterion of division it is relatively simple to classify all neutrophils in a well made film. Cooke and Ponder recognize 5 groups of neutrophils having from 1 to 5 distinct lobes in the nucleus. While this is a simplification of Arneth's classification, the emphasis is






Class I	
II	
III	
IV	
V	

FIG. 57.—COOKE AND PONDER'S CLASSIFICATION OF THE NEUTROPHIL (HADEN)

placed on subdivision of the segmented or more mature cells as in Arneth's method, rather than on the increase of more immature cells. It is desired to know the degree of immaturity of the neutrophils rather than the subdivisions of maturity. Pernicious anemia is one of the few clinical conditions in which the appearance of a larger number of very mature neutrophils or "shift to the right" is of diagnostic importance. Some "shift to the left" is encountered in the presence of almost every infection, and often in other toxic conditions.

Farley, St. Clair and Reisinger<sup>6</sup> have suggested a simpler classification of the neutrophils into *nonfilament* and *filament* types (Fig. 58 and Plate 1) which supplies all essential and practical information required on the degree of immaturity of these cells. As stated by these investigators, "The test of a given clinical procedure is often

the ease of its application divided by its usefulness". According to this classification nonfilamented neutrophils embrace all those in which the lobes are connected by broad dense bridges of chromatin material. Under the "nonfilamented" they include the juvenile and stab cells of Schilling, now commonly designated as metamyelocytes, meaning "after myelocyte", which seems to express the immaturity of the cells very clearly. Filamented neutrophils are those in which 2 or more lobes are connected by a fine thread-like filament of chromatin material. Sometimes a separate and distinct lobe is present but the filament cannot be seen. They should be classified as filamented. These filamented cells are also known as "segmented neutrophils" and represent mature cells, the degree of maturity being indicated by the number of lobes, as pointed out by Arneth and Schilling.

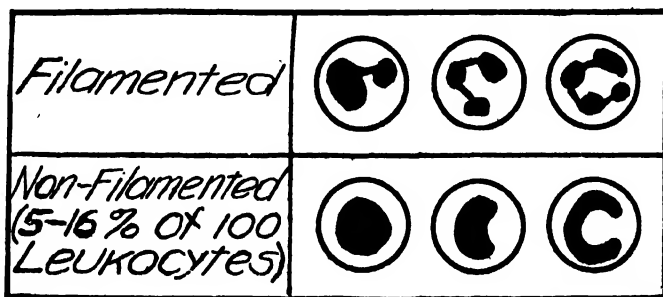


FIG. 58.—CLASSIFICATION OF THE NEUTROPHILS  
According to Farley, St. Clair and Reisinger (Haden)

**Schilling Index.**—According to this method of classification not more than 16 per cent of the neutrophils should be nonfilamented, or immature, under normal conditions. Any figure above this indicates an increase in the degree of immaturity, suggestive of infection and constituting a "shift to the left". This may be expressed according to the Schilling index in terms of the following fraction:

$$\frac{\text{Per cent or number of immature or nonfilamented neutrophils}}{\text{Per cent or number of mature or filamented neutrophils}} = \text{Schilling Index.}$$

Another method of expressing the shift is by dividing the mature (filamented) forms by the immature (nonfilamented) forms (nuclear index). This is the same as the Schilling index inverted and so expresses the index as a whole number.

$$\frac{\text{Per cent or number of mature neutrophils}}{\text{Per cent or number of immature neutrophils}} = \text{Nuclear Index.}$$

Either of these indices may be used to express the shift obtained with any of the classifications described. However, the normal limit will vary. Schilling gives as normal, for his index, any figure over 1/13 which corresponds to a nuclear index of 13. When using the Schilling index the higher the number the greater the shift to the left and when the inverted index is used, the lower the number the greater the shift to the left.

**Basophilic Granulation of Neutrophils (Toxic Granulation and Degenerative Changes).**—Of equal importance with the classification of the neutrophils in relation to differential leukocyte counts, is a study of these cells for changes probably due to the effect of various toxins upon the leukoblastic tissues of the bone marrow during

the course of various acute and chronic infections. These changes may not only affect the cell as a whole or the cytoplasm, but the nucleus and especially the granules (Fig. 59).

These cells are likely to be unduly fragile which sometimes leads to their crushing and disintegration in the preparation of blood films. They may appear large, swollen and ovoid in shape with a "moth-eaten" or frayed appearance and a tendency to form pseudopods.

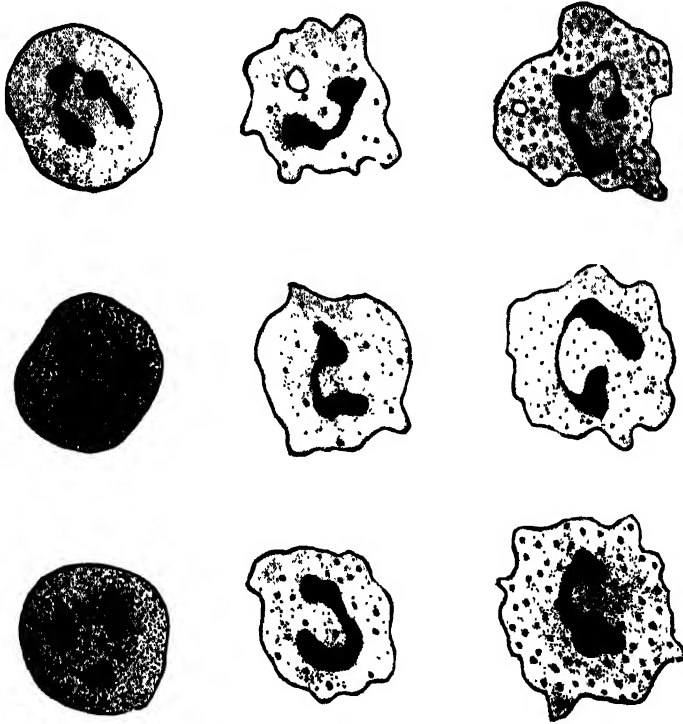


FIG. 59.—TOXIC CHANGES IN NEUTROPHILS

The *cytoplasm* is likely to show varying degrees of vacuolization. Normally, only an occasional neutrophil will show the presence of a vacuole but in degenerated cells they may be numerous, appearing as regular, round, clear spaces.

The pattern of the *nucleus* may vary from normal, with irregularity in staining rendering it hyperchromatic, pyknotic or structureless. In some instances the nucleus appears swollen with an irregular jagged outline and cloudiness of the chromatin.

The *granules* in the cytoplasm are numerous, small, of uniform size and pinkish in color under normal conditions. Basophilic granulation is manifested by the presence of basophilic granules of a deep blue color. They may be small and scattered among the normal pinkish granules or large, coarse and irregular in distribution, in which case few, if any, normal granules are to be seen. In some cells they may be so large that the cells resemble basophils. These toxic granules may be present in both young or nonfilamented and mature or filamented neutrophils. This change is often referred to as *toxic granulation*.

Under normal conditions the granules are sharply defined when stained by the

peroxidase method. Basophilic or "toxic granules", however, are not brought out by this method of staining. In other words, basophilic granules in neutrophils apparently represent an interference or failure in the development of granules in neutrophils in the bone marrow as the result of infection and disease.

These basophilic granules are readily stained by the Wright or Giemsa methods. They are likewise readily stained by fixing the blood film with absolute methyl alcohol for 2 minutes, drying in the air, staining with Löffler's methylene blue for 2 minutes, washing in water and drying. Normal granules are unstained or appear as a very few fine pinpoint granules. Basophilic or toxic granules, however, appear dark blue or blue violet in a dirty gray or blue-gray cytoplasm.

**Degenerative Index.**—In making differential leukocyte counts in cases of known or suspected infections it is advisable, therefore, to report on the presence or absence of degenerated neutrophils. The percentage of degenerated cells may be recorded or the results expressed in terms of the "degenerative index" of Kugel and Rosenthal.<sup>7</sup> This index is calculated by dividing the number of neutrophils showing basophilic granules, by the total number of neutrophils studied as follows:

$$\frac{\text{Number of neutrophils showing basophilic granules}}{\text{Total number of neutrophils}} \times 100 = \text{Degenerative Index}$$

Example: Total neutrophils studied: 50.

Number showing degeneration or basophilic granules: 28.

$$\frac{28}{50} \times 100 = 56 \text{ per cent (degenerative index)}$$

**Döhle's Inclusion Bodies.**—Döhle's inclusion bodies (Fig. 60) are found in the cytoplasm of the neutrophils in the majority of cases of scarlet fever and sometimes in diphtheria, pneumonia, and other infectious diseases, but never in German measles and rarely in measles. They occur as pear-shaped short rods and are readily seen in blood films stained according to the method of Wright or Giemsa. Prolonged staining with pyronine-methyl green (Pappenheim method), however, is best as the bodies stain bright red while the nuclei are purplish.



FIG. 60.—DOHLE INCLUSION BODIES  $\times 1500$ . (Wood.)

## ABNORMAL VARIETIES OF LEUKOCYTES

The following leukocytes are not found in the circulating blood in health. Indeed, except when activated by disease, the younger forms are even rare in the bone marrow. At birth all the bones contain active bone marrow but in the healthy adult only the flat bones, such as the scapulae and the ribs and the bodies of the vertebrae, are characterized by formative marrow. Cells of 2 series are encountered as follows (Table 6, page 89):

**Granulocytic Series.**—1. *Myeloblasts.*—These are the youngest or stem cells of this series. They are slightly larger than the well known neutrophil leukocyte (14 to 20 microns) and possess a deep blue basophilic cytoplasm when stained with Wright's stain. There are no cytoplasmic granules. The nucleus is characterized by fine chroma-

tin markings in the form of a fine stippling. To some observers the spaces between the chromatin markings suggest a fine sieve-like character. The nucleus stains bluish purple by Wright's method. There are several small nucleoli which are stained light blue (Plate I).

2. *Promyelocytes*.—As the myeloblast matures, it acquires cytoplasmic granules. It is then called the promyelocyte (10 to 16 microns). At first these granules are of the azurophile variety, so called because with Wright's stain they take a rich blue stain. They are not affected by the oxidase stain of Goodpasture and although they are generally large granules, they vary much in size. The cytoplasm is less basophilic than in the myeloblast (Plate I). The nucleus stains somewhat more deeply and the chromatin particles seem somewhat coarser. As this cell grows older, the azurophile granules are replaced by specific granules which become black when treated with Goodpasture's oxidase stain. It is at this point that the cell differentiates into 1 of the 3 following specific types of myelocytes: neutrophilic, eosinophilic or basophilic.

3. *Neutrophilic Myelocytes*.—The cytoplasm has at this stage reached its complete development and acquired phagocytic powers. The cytoplasm is very lightly acidophilic and packed with small, not easily seen, violet granules (Wright's stain). The cell is slightly smaller than the myeloblast (8 to 12 microns). The nucleus is still round but it is more deeply stained, its chromatin markings are coarser and nucleoli are rarely found. Further maturity of the cell is shown not by cytoplasmic changes but by changes in the nucleus. The round form becomes indented (juvenile leukocyte), the indentation extends deeper (stab leukocyte) and finally segmentation occurs (segmented leukocyte). During this process of indentation and segmentation, the chromatin condenses so that it stains more deeply from blue-red to bluish-black; the chromatin masses and nuclear membrane become thicker, and the spaces between the chromatin markings become wider (Plate I).

4. *Eosinophilic Myelocytes*.—This cell develops granules which are much larger than the neutrophilic granules and takes a distinct red, sometimes brownish-red stain (Wright's stain). The nuclear changes of this cell are similar to those of the neutrophil and the same type cells are formed (Plate I).

5. *Basophilic Myelocytes*.—In this cell the granules are also large like those of the eosinophil but they stain a deep blue although an occasional metachromatic (red or bluish-red) granule is encountered. Nuclear maturity occurs as in the other forms of myelocytes. In active bone marrow, cells of varying maturity are seen from the most immature to the mature segmented form. They are studied best in films prepared by smearing the marrow on a slide and staining in the same manner as blood films (Plate I).

**Lymphoid Series.**—Cells of this series may be found in the bone marrow as well as in the circulating blood in leukemic states. Normally they are found in lymphoid tissue.

1. *Lymphoblasts*.—This cell closely resembles the myeloblast in appearance although smaller (12 to 18 microns) with no granules in the cytoplasm. It may be found in the germinal centers of lymph nodes in health.

2. *Young Forms of Lymphocytes*.—These are characterized by delicately stained nuclei of a vesicular character. They do not contain nucleoli. They are usually large cells from 10 to 20 microns in diameter. The cytoplasm, like that of the mature lymphocyte, is lightly basophilic. These young lymphocytes are frequently incorrectly

called monocytes because of their delicate nuclear structure. They are found characteristically in infectious mononucleosis. Young lymphocytes may or may not possess azurophilic granules.

3. *Türk's Irritation Forms*.—The true nature and significance of these cells is disputed. The nucleus stains deeply, is round with irregular markings, and the cytoplasm is intensely basophilic (Plate I).

**Other Series.**—*Monoblasts*, or *promonocytes*, are derived from reticulo-endothelial cells and are difficult to distinguish from myeloblasts and lymphoblasts except by the trend of the blood picture (Table 6).

*Plasma cells* vary in diameter from 8 to 16 microns (Plate I). The cytoplasm is abundant, deeply basophilic, and free of granules although they sometimes show the presence of azurophilic granules. The nucleus is usually oval and smooth with a coarse cartwheel arrangement of the chromatin. Nucleoli are absent. There is almost always a bright, perinuclear sphere. Plasma cells are oxidase-negative and may be confused with erythroblasts. They may originate in lymphoid or myeloid tissues.

## NORMAL AND ABNORMAL ERYTHROCYTES

The erythrocytes of stained blood smears should also be examined and reported upon, especially in the anemias.

1. *Normal erythrocytes* or *normocytes* are acidophilic and of nearly uniform size, averaging 7.6 microns in diameter (Plate II). There may be an occasional cell 5.5 or 9.5 microns in diameter. The center of each is somewhat paler than the periphery. In stained preparations distorted shapes may be seen due to mechanical distortion in preparing smears. In wet preparations they may occur in *rouleaux formation* and show some *crenation*. Neither of these changes possesses pathological significance. *Endoglobular degenerations* may be observed in the larger erythrocytes (macrocytes). They are evidenced by rounded or irregular, sharply or indefinitely outlined colorless areas (Plate II) and should not be confused with the highly refractile colorless spots so frequently seen when blood films are dried too slowly.

2. *Poikilocytes* are oval, pyriform, caudate, saddle-shaped and club-shaped erythrocytes (Plate II). They may be large or small and are seen in any severe anemia not of the aplastic type. Their presence constitutes *poikilocytosis*.

3. Abnormal variations in size is called *anisocytosis*. When the erythrocytes are smaller than normal (5 microns or less in diameter) they are called *microcytes* (Plate II) and when larger (10 to 12 microns) *macrocytes* (Plate II). Extremely large forms (12 to 16 microns in diameter) are called *megalocytes* (Plate II) and still larger ones *gigantocytes* (16 to 25 microns in diameter).

4. *Sickle-shaped erythrocytes* (Plate II) are characteristic of sickle-cell anemia which occurs almost exclusively in the Negro race. They are seen best in wet preparations of the blood prepared by special methods.

5. "*Target*" *erythrocytes* are so called because of the presence of dark areas in the centers of the cells surrounded by light zones (Plate II). They are also known as "Mexican hat" or "sugar-loaf" cells. They are transitory in acute posthemorrhagic anemias but characteristically present in sickle-cell anemia, Cooley's anemia and other anemias in response to blood destruction rather than a cause of it.

6. *Ovalocytes* are elliptically shaped erythrocytes, likewise best seen and studied



in wet unstained preparations of fresh blood (Plate II). Their presence constitutes *ovalocytosis* which is an hereditary anomaly with no tendency to anemia in most cases. The cells are oval or rod-shaped.

7. *Spherocytes* are shorter and much thicker than normal erythrocytes. They occur in congenital hemolytic jaundice and are more readily hemolyzed in hypotonic saline solution.

8. In the anemias with diminished hemoglobin, the central pale areas of erythrocytes become larger and paler, constituting *achromia*. In extreme instances the periphery retains a fairly deep color so that the cells become mere rings, the so-called "pessary forms".

9. *Polychromatophilia* is the term used for indicating the abnormal affinity of erythrocytes for the basic stains (Plate II). When present, many erythrocytes will be seen taking the basic blue stain in varying degrees, usually pale to light blue instead of pale pink. The condition is abnormal and found in anemias where there is active regeneration of erythrocytes.

10. *Basophilic degeneration*, or "*stippling*", is a condition in which there are many very fine to coarse blue dots or granules present in the erythrocyte (Plate II). They are found in cases where erythrocytic regeneration is active and probably represent cells which have undergone a degenerative change before they were fully mature. Their presence in suspected cases of lead poisoning is of diagnostic value.

11. *Reticulated erythrocytes* are young cells which, when stained with brilliant cresyl blue, show filaments which are well stained. The method of Osgood and Wilhelm is recommended. Although these filaments take the basic stain, they will not stain in the usual dry smear. They are often arranged in skeins or wreaths (Plate II).

12. *Nucleated erythrocytes* are immature cells which are thrown into the circulation in severe anemias and leukemias in which there is active regeneration of bone marrow. They are classified according to their size as follows:

(a) *Normoblasts*, which are 7 to 8 microns in diameter and contain 1, rarely 2, small, round, sharply defined nuclei which are deeply stained (Plate II). As a result of degenerative changes, however, the nuclei may be irregular in shape, cloverleaf forms being common. In some instances they may be completely broken up into fragments and constitute the so-called nuclear particles or Howell-Jolly bodies, of which all but 1 or 2 have disappeared from the cell.

(b) *Microblasts*, which are 5 microns or less in diameter (Plate II).

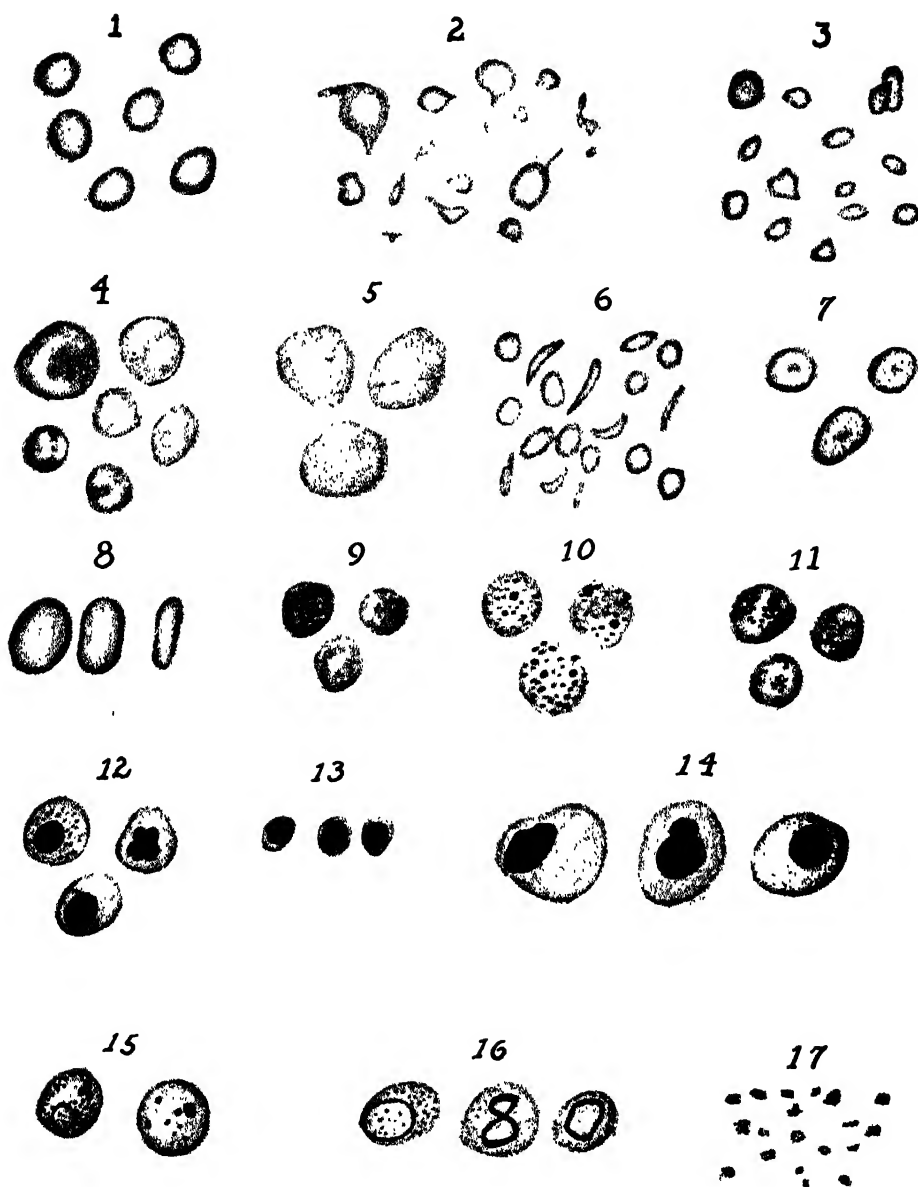
(c) *Macroblasts*, which are younger cells than normoblasts and 10 to 12 microns in diameter (Plate II). The nuclei are large, oval and rather palely stained with delicately stained chromatin networks showing large and numerous openings.

(d) *Megaloblasts*, which are still larger than macroblasts with a diameter of 12 to 16 microns and showing a more delicately stained chromatin network. *Gigantoblasts* are very large megaloblasts measuring 16 to 25 microns in diameter.

13. As previously stated, *Howell-Jolly bodies* are supposed to arise from the nuclei of erythrocytes through karyorrhexis and karyolysis (Plate II). These peculiar nuclear remains vary in size down to very fine fragments easily confused with basophilic stippling.

14. *Cabot's ring bodies* are ring or figure-of-8 structures which stain reddish blue, red, or blue with Wright's stain. Cabot<sup>8</sup> regarded them as remnants of incompletely

## PLATE II



NORMAL AND ABNORMAL ERYTHROCYTES AND PLATELETS (WRIGHT'S STAIN)

1, Normal erythrocytes (normocytes); 2, Poikilocytes; 3, Microcytes; 4, Macrocytes; three cells show endoglobular degeneration; 5, Megalocytes; 6, Sickie cells; 7, Target cells; 8, Ovalocytes; 9, Polychromatophilia; 10, Basophilic degeneration ("stippling"); 11, Reticulocytes; 12, Normoblasts; 13, Microblasts; 14, Macroblasts; 15, Howell-Jolly bodies; 16, Cabot's ring bodies; 17, Normal platelets.



absorbed nuclei. Schleicher,<sup>9</sup> however, has recently shown that they are neither nuclear remnants nor identical with the nuclear membrane, but merely laboratory creations, the result of cellular degenerations induced by hemolytic agents, *i.e.*, aggregated and denatured colloid proteins.

## METHOD FOR DIFFERENTIAL COUNTING OF LEUKOCYTES

**Principles.**—Differential leukocyte counts are made for the purpose of determining the percentages of normal and abnormal leukocytes, or their numbers per c.mm. of blood. The latter is preferred. *The erythrocytes should be examined at the same time and all abnormal types reported.*

**Procedure.**—1. First, inspect the blood film under low magnification to determine if the leukocytes are well distributed. If not, a better slide should be chosen.

2. If the film proves satisfactory it should be examined systematically with an oil-immersion objective and each leukocyte recorded. The Marbel blood cell calculator (Fig. 23) is very convenient for this purpose. At least 4 different areas of the slide should be examined, preferably the margins of the heavy and thin portions of the film well into the center of the slide. Large leukocytes, like the neutrophils, tend to accumulate at the edges of a film and the smaller ones (lymphocytes) nearer the central portions.

3. The differential count should show the actual numbers or percentages of the following normal leukocytes: (a) Lymphocytes; (b) monocytes; (c) nonfilament neutrophils (juvenile and stab metamyelocytes); (d) filament neutrophils, (e) eosinophils and (f) basophils. The Schilling index should be determined, especially if the immature neutrophils are increased above normal.

4. It is also advisable to determine the presence or absence of degenerated neutrophils. If these are present, the "degenerative index" should be reported.

5. The differential count should also show the actual numbers or percentages of the following abnormal leukocytes, if any are present: (a) Myeloblasts; (b) promyelocytes; (c) myelocytes (neutrophilic, eosinophilic, basophilic); (d) lymphoblasts; (e) plasma cells; (f) monoblasts and (g) Türk irritation cells.

6. As stated previously, the erythrocytes should be reported upon at the same time and especially the presence of any abnormal cells embracing (a) achromia; (b) polychromatophilia; (c) basophilic degeneration or "stippling"; (d) poikilocytes; (e) microcytes, macrocytes or megalocytes; (f) normoblasts, microblasts or macroblasts and (g) such unusual cells as sickle erythrocytes, "target" erythrocytes, spherocytes, ovalocytes, and Howell-Jolly bodies.

7. For accurate work at least 400 leukocytes should be counted. Under routine conditions, however, the counting of 100 or 200 cells is sufficient. If a total leukocyte count has been made beforehand it is recommended that the number of leukocytes to be classified under routine conditions may be as follows:

For total counts under 10,000, classify 100 cells.

For total counts of 10 to 15,000, classify 200 cells.

For total counts of 15 to 20,000, classify 300 cells.

For total counts of 20 to 25,000, classify 400 cells.

For total counts over 25,000, classify 500 cells.

**TABLE 6.—GENERAL CHARACTERISTICS OF THE LEUKOCYTES (WRIGHT'S STAIN)**

Name	Size (Microns)	Nucleus			Cytoplasm	Granules
		Nucleoli	Shape	Chromatin		
Lymphocyte	7-15	None	Round or indented	Blotchy	Robin's egg blue Deeply basophilic	Comparatively rare Azurophilic
Monocyte (Endothelial leukocyte)	9-13	None	Indented or may appear round	Thready	Grey-blue Basophilic	Azurophilic if any
Juvenile (Nonfilament) (Metamyelocyte)	8-12	None	Indented Less than 50 per cent invagina- tion	Scale-like	Reddish-blue Weakly neutrophilic, eosinophilic or basophilic	Neutrophilic Eosinophilic Basophilic
Stab (Nonfilament) (Metamyelocyte)	8-12	None	Rod-shaped More than 50 per cent invagina- tion	Scale-like	Reddish-purple Weakly neutrophilic, eosinophilic or basophilic	Neutrophilic Eosinophilic Basophilic
Neutrophil (Filament) (Segmented)	8-10	None	Lobed	Distinct scales	Reddish-purple Neutrophilic	Neutrophilic
Eosinophil	7-10	None	Indented or lobed	Distinct scales	Reddish Weakly basophilic	Eosinophilic
Basophil	7-10	None	Indented or lobed	Indistinct scales	Bluish Weakly basophilic	Basophilic

Normal

Abnormal						
Myeloblast	14-20	4-6	Round	Finely stippled	Bluish-purple Deeply basophilic	None
Promyelocyte	10-16	1-6	Round	Finely stippled	Bluish-purple Faint bluish purple Reddish purple	Neutrophilic Basophilic Eosinophilic Azurophilic
Myelocyte	8-12	None	Round	Scale-like	Bluish-purple Faint bluish-purple Reddish purple	Neutrophilic Basophilic Eosinophilic
Lymphoblast	12-18	4-6	Round	Distinctly stippled	Bluish-purple Deeply basophilic	None
Plasma cell	8-16	None	Oval	Cart-wheel	Dark blue Intensely basophilic	Extremely rare Azurophilic
Türk cell	7-10	None	Round	Dark and patchy	Dark blue Intensely basophilic	None
Monoblast (Promonocyte)	12-18	4-6	Round	Stippled	Bluish-purple Deeply basophilic	None

8. When the total leukocyte count is 1000 or less it may be necessary to examine several slides before 100 cells are classified. To save time, oxalated venous blood (page 48) may be used providing blood films are prepared almost immediately after the collection of blood before the leukocytes have become distorted and difficult to classify. The method is as follows: (a) Centrifuge the oxalated blood at high speed for 10 to 15 minutes; (b) with a pipet remove the supernatant plasma down to the thin whitish layer of leukocytes which overlies the erythrocytes; (c) with the same pipet, remove and transfer small drops of the leukocyte layer to slides and prepare films in the same manner as of whole blood; (d) dry quickly and stain according to the Wright method.

9. If the total number of leukocytes classified is an even number like 100, 200, 300, 400 or 500, the percentage of an individual cell is readily determined or Waugh's *White Blood Cell Differential Tables* \* may be used. If, however, the total cells classified is an odd number, the percentage of an individual cell may be calculated according to the formula: total cells classified is to an individual cell as 100 is to X, as per the following example:

Total cells classified .....	116
Neutrophils .....	92
$9200$	
$X = \frac{9200}{116} =$	79.3 per cent neutrophils

10. However, as the percentages of the various leukocytes do not indicate their actual numbers per c.mm. of blood, it is advisable (and recommended) to convert them into the numbers of each type per c.mm. of blood, although the medical profession as a whole is not yet acquainted with the normal figures with which to interpret reports. The *per cent of any type of cell may be increased or decreased without any change in the actual number* by a change in the total leukocyte count. Thus a total leukocyte count, in an adult, of 4000 with 90 per cent neutrophils would seem to indicate a leukopenia with a neutrophilia, whereas the actual number of neutrophils is 3600 per c.mm. of blood, which is within normal. On the other hand, a total leukocyte count of 20,000 with 18 per cent neutrophils would seem to indicate a leukocytosis with a neutropenia whereas the neutrophils are actually 3600 per c.mm. of blood, which is likewise within normal. Consequently, the percentage system as ordinarily employed, is subject to gross errors in the interpretation of differential leukocyte counts and should be abandoned. Probably the best plan at the present time is to report both the total numbers of the various leukocytes per c.mm. of blood and their percentages. The former is readily calculated from the total leukocyte count. If, for example, the latter is 12,500 with 77 per cent neutrophils, the actual number of these cells per c.mm. of blood would be  $125 \times 77$  or 9625.

**Normal Differential Leukocyte Counts According to Age.**—The total leukocytes of infants and children up to at least 10 years of age is normally higher than in adults. The absolute numbers and percentages of the individual cells are likewise different, as shown in Table 3, mainly due to a larger number of lymphocytes and a smaller number of filamented or mature neutrophils. Thus, at birth, about 12,000 neutrophils and 3000 lymphocytes are usually found per c.mm. of blood. By the sixth

\*D. Appleton-Century Co., New York.

day the lymphocytes are around 10,000 followed by a gradual decline, reaching the normal for adults at about puberty. By the ninth day the neutrophils average 2000 to 7000 on through to adult age. There are no essential differences according to sex in either children or adults.

In general terms the ranges of absolute numbers per c.mm. of blood and percentages of the individual cells in *infants* and *children* under normal conditions are shown in Table 7 while those for *adolescents* and *adults* are shown in Table 8. The percentages shown in Tables 7 and 8 are those usually encountered in normal differential counts. In these tables, the neutrophils include both the mature and immature forms. These may be counted separately for the purpose of determining shifts.

**TABLE 7.—NORMAL DIFFERENTIAL LEUKOCYTE COUNTS IN CHILDREN**

Cells	3 months to 3 years	3 years to 5 years	5 years to 15 years
Neutrophils	2000-7000 (40-50%)	3000-8000 (50-60%)	3000-7000 (55 to 65%)
Eosinophils	25-700 (0.5-5%)	50-700 (1-5%)	50-500 (1 to 5%)
Basophils	0-50 (0-0.5%)	0-50 (0-0.5%)	0-50 (0 to 0.5%)
Lymphocytes	4000-9000 (50-60%)	2500-6000 (40-50%)	1500-4500 (30-40%)
Monocytes	25-700 (0.5-5%)	25-700 (0.5-5%)	25-600 (0.5-6%)

**TABLE 8.—NORMAL DIFFERENTIAL COUNTS IN ADOLESCENTS**

Cells	15 years and over
Neutrophils	3000-7000 (60-70%)
Eosinophils	50-400 (1-4%)
Basophils	0-50 (0-0.5%)
Lymphocytes	1000-3000 (20-30%)
Monocytes	100-600 (2-6%)

As an example of a "shift to the left" or an increase of metamyelocytes (non-filamented neutrophils) the following count from a case of acute suppurative appendicitis in an adult with a total leukocyte count of 15,000 may be given:

Nonfilament neutrophils	2100 (14 per cent)
Filament neutrophils	10,425 (69.5 per cent)
Eosinophils	0
Basophils	75 (0.5 per cent)
Lymphocytes	1800 (12 per cent)
Monocytes	600 (4 per cent)

In the above, the proportion of nonfilamented neutrophils to the filamented neutrophils is 1 to 5, which indicates a definite *shift to the left*.



It should be remembered that it is *neither the percentage nor the number of immature neutrophils that determines the shift to the left, but rather the proportion of the immature to the mature forms*. For example, a count with 5 per cent nonfilament forms with 40 per cent mature or filamented forms would be a shift to the left. However, if there were 80 per cent mature forms then there would not be a shift. If there were 400 filamented or immature forms with 8000 mature forms, there would be no shift to the left, but if there were only 2000 mature forms then there would be a definite shift to the left. The proportion according to Schilling is 1 to 13+. This proportion will vary according to the criteria used for classifying the cells; for example, if the filament-nonfilament method of Farley is used, the proportions will be different.

### METHOD FOR DETECTION OF SICKLE ERYTHROCYTES

1. In active sickle cell anemia the characteristic erythrocytes may be discovered in the course of routine examinations. However, when the disease is suspected the examination of wet preparations is required as follows: (a) Place a small drop of blood on a slide and add a drop of isotonic saline solution; (b) cover with a cover-glass to secure a thin preparation; (c) seal the preparation with vaselin and keep at room temperature; (d) examine at intervals up to 24 hours, and if necessary up to 3 days, before discarding as negative.

2. The following method of Beck and Hertz<sup>10</sup> is recommended for the detection of the sickle cell trait:

(a) Place from 0.2 to 0.5 cc. of saline citrate solution (3 per cent sodium citrate in normal saline) in a test tube.

(b) Clean and prick the finger as in collecting blood for a count.

(c) Collect 1 or 2 drops of blood in the tube containing the saline citrate. Invert and mix.

(d) Cover with sufficient oil to make a layer 1 cm. thick. Make sure no bubbles of air are under the oil.

(e) Let the preparation stand at room temperature for 24 hours; then introduce 0.2 to 0.5 cc. of formalin solution (0.85 gm. of sodium chloride to 1000 cc. of 10 per cent neutral formalin) beneath the oil layer with a pipet. Thoroughly mix by forcing the liquids in and out of the pipet several times. Do not break up the oil layer for fear of letting air in too soon. Two or 3 minutes or more should be allowed for fixation. After this period the suspension is mixed again with the pipet to insure a uniform distribution of cells.

(f) Remove a few drops from the tube, wipe away the excess oil from the tip, and place a drop on a glass slide.

(g) Cover and examine.

(h) The percentage is calculated in the manner of the differential leukocyte count.

The saline-citrate, the paraffin oil, and the formalin are to be kept in separate bottles, stoppered and labeled. One pipet is to be used for handling the saline-citrate solution, one for the paraffin oil, and one for the formalin. Mark these pipets as a trace of formalin in the blood prior to sickling may prevent the deformity.

Permanent preparations can be made by making smears; air dry and fix in flame. Stain with 1 per cent aqueous solution of fuchsin. Formalin treated cells do not stain well with Wright's stain.

## METHODS FOR MEASURING ERYTHROCYTES AND DETERMINING THE MEAN CELL DIAMETER

**Principles.**—The following methods are used in determining the diameters of erythrocytes: (a) By projection and direct measurement of tracings of the projected image, as in the method of Price-Jones; <sup>11</sup> (b) by measurement of photomicrographs; (c) measuring with an eyepiece micrometer; (d) measuring with a filar micrometer; (e) by use of the diffraction method of Pijper <sup>12</sup> and (f) by use of a diffraction method employing the Haden-Hausser erythrocytometer. In the Haden method <sup>13</sup> the *mean cell diameter* is read off on the scale of the Haden-Hausser erythrocytometer located on the wheel used to rotate the pinion. In some other methods it is calculated by multiplying each diameter in microns by the number of cells found with that diameter, adding all the products and dividing by the total number of cells measured.

**Method of Price-Jones.**—1. Prepare smears in the same manner as for differential counting. The films should be thin — the thinner the better — dry in air without heat.

2. Stain with Jenner's stain for 2 minutes, and after washing with distilled water and drying, superstain with weak aqueous solution of eosin for 2 minutes. These details should always be adhered to since it is found that alterations in the fixing and staining reagents can produce changes in the mean diameters of the cells.

3. Adjust some simple form of projection apparatus for a magnification of 1000 diameters and project the microscopic field onto a sheet of paper lying on the table. Outline in pencil the red cells. Two diameters, maximum and minimum, of each cell are then measured to 0.5 mm. with a glass millimeter scale and may be directly expressed in terms of microns; the mean of these 2 measurements is accepted as the diameter value of the cell. The mean diameter of several hundred cells should be taken to represent the mean diameter for any sample of blood.

**Method of Haden.**—This method employs the Haden-Hausser erythrocytometer (Fig. 61) and is preferred; the technic is as follows:

1. Prepare thin, uniform films of blood on coverglasses and stain with Wright's stain. The cells in the film employed should touch but not overlap.

2. Place a few drops of 0.9 per cent sodium chloride solution on the dry stained film and invert the coverglass on a clean glass slide. If the film is made on a slide and stained, an area should be selected for reading where the film is satisfactory and the shape of the cells is unaltered.

3. Place the preparation in the instrument and move it up and down until the inner red ring is centered over the inner set of holes (Fig. 62). Three sets of apertures are provided. For reading with the outer set, the second red ring is used; for the middle set, the yellow ring. In a normal blood, the readings should be the same for

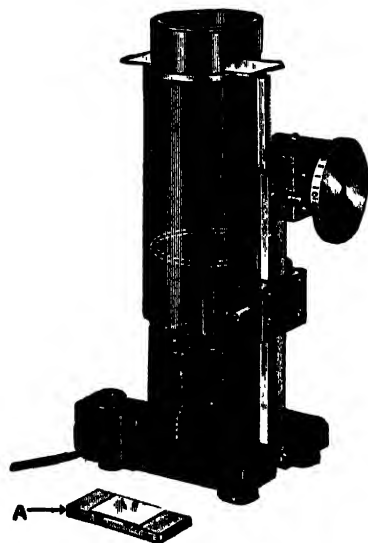


FIG. 61.—HADEN-HAUSSER ERYTHROCYTOMETER

all 3 rings and apertures. In an abnormal blood, this is not necessarily true. The measurement of the *mean cell diameter* is always made with the inner red ring centered over the inner set of apertures. This mean cell diameter is read off on the scale located on the wheel used to rotate the pinion. Fixed unstained films on slides may also be used; likewise fresh, wet unstained preparations of oxalated blood (requires a special chamber). Detailed directions accompany the instrument.

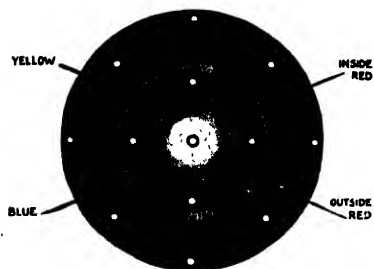


FIG. 62.—FIELD OF VIEW IN THE HADEN-HAUSER ERYTHROCYTOMETER

4. The average diameter of erythrocytes is 7.5 microns with a range of 7.0 to 8.0 microns. A few cells may be smaller than 6.0 or larger than 9.0 microns. The average mean diameters of normal cells by the Haden method is 7.3 to 8.0 microns. An *increase* of the mean diameter is characteristic of pernicious anemia and a *decrease* of the iron deficiency anemias. In hemolytic jaundice the mean diameter is also decreased but with normal color, mean corpuscular volume and saturation indices because the cells are spherical and thicker, as determined by the mean corpus-

cular thickness. Aside from hemolytic jaundice, however, all essential clinical data on the size of erythrocytes is obtained by determining the mean corpuscular volume.

**Method of Nicholson.**—1. Place an ocular micrometer in the eyepiece of the microscope by unscrewing the top lens and placing the micrometer so it will rest on shelf of eyepiece. Then replace lens.

2. Under oil immersion, bring into focus one of the smallest squares in the central square millimeter of the hemocytometer.

3. Adjust the tube length of the microscope so that the distance encompassed by 50 graduations of the micrometer coincides with the distance across the smallest hemocytometer square which is 50 microns across. In this position, 1 division of the micrometer is equal to 1 micron when using the oil immersion lens.

4. Prepare and stain a thin blood smear in the usual manner.

5. Remove the hemocytometer and examine the smear under oil immersion. Measure to the nearest 0.5 microns at least 200 erythrocytes selected from different areas of the smear.

6. Record by tally each erythrocyte according to its diameter and total the cells in each group.

7. Plot a curve using the diameter in microns as the abscissa and the number of cells as the ordinate of the graph.

The normal curve is an acute angle with slightly outcurving base. The peak is between 7.0 and 8.0 microns. In microcytic anemia the peak is to the left and is less acute. In macrocytic anemia the peak is the right of normal and is less acute and the base is wide.

### METHOD FOR DETERMINING THE VOLUME THICKNESS INDEX (HADEN)

**Principles.**—The volume thickness index increases with any tendency to a globular form or the spherocytosis of erythrocytes. This index remains constant with constant erythrocyte shape, independent of changing erythrocyte size. The determination

is of special value in the diagnosis of congenital hemolytic jaundice in which the mean diameter of the erythrocytes is decreased, but the mean corpuscular volume within normal because of the increased thickness of the erythrocytes. The volume thickness index may be determined by dividing the mean corpuscular volume of the patient's blood by the calculated mean corpuscular volume corresponding to the measured mean erythrocyte diameter.<sup>14</sup> The technic is as follows:

- Procedure.**—1. Determine the mean corpuscular volume of the patient's blood.  
2. Determine the mean diameter of the patient's erythrocytes.  
3. Take the normal mean corpuscular volume as 90 cubic microns.  
4. Take the calculated mean corpuscular volume corresponding to the mean diameter of erythrocytes as follows: <sup>14</sup>

<i>Mean Diameter</i>		<i>Calculated Mean Corpuscular Volume</i>
6.0 microns	=	0.48
6.5 "	=	0.60
7.0 "	=	0.75
7.5 "	=	0.90
8.0 "	=	1.10
8.5 "	=	1.48
9.0 "	=	1.60

5. Volume thickness index =  $\frac{\text{Mean corpuscular volume observed}}{90 \times \text{mean corpuscular volume calculated}}$   
6. Calculate as per the following example:

Mean corp. vol. of patient's blood.....	81	cubic microns
Mean dia. of patient's erythrocytes.....	6.5	microns
Calculated mean volume corresponding to 6.5 microns.	0.60	
81		
$\frac{81}{90 \times 0.60} = 1.50$	Volume Thickness Index	

7. The normal volume thickness index is 1.00. Haden states that typical volume thickness indices are as follows: Pernicious anemia, 0.96; microcytic anemia, 0.91; obstructive jaundice, 0.75; chronic hemolytic jaundice, 1.92.

## METHODS FOR COUNTING RETICULOCYTES

1. Prepare the stain by dissolving 1.0 gm. brilliant cresyl blue (Grubler) in 100 cc. of normal saline solution. Filter. The stain keeps well.
2. Draw the stain in a leukocyte counting pipet to the mark 1.0.
3. Draw in an air bubble and then capillary blood from a finger or the lobe of an ear to the mark 1.0.
4. Draw the blood into the bulb of the pipet and mix thoroughly with the stain by rotating and shaking.
5. Allow to stain in the pipet for 5 minutes.
6. Prepare a film on a slide in the same manner as in the preparation of blood films for differential leukocyte counts.

7. Allow to dry and examine the erythrocytes with an oil immersion objective. The reticulum stains a pale blue color (Plate II). The film may be counterstained with Wright's stain but this is not necessary. A restrictor eyepiece is advisable.

8. Examine 1000 erythrocytes. Record the number of reticulocytes and divide by 10 to obtain the percentage. The normal varies from 0.5 to 1.0 per cent.

An *alternate method* is as follows: 1. Smear across a slide a few drops of a 1 per cent alcoholic solution of brilliant cresyl blue as in preparing a blood film.

2. Dry in the air.

3. Place a drop of blood in the center of a coverglass and place blood side down on the dried stain.

4. Let stand 10 minutes.

5. Examine with an oil immersion objective. The thinner the preparation, the easier the count.

6. Count 1000 erythrocytes, noting the number containing the bluish strands of reticulum. Divide this number by 10 to obtain the percentage of reticulocytes.

**Direct Smear Method.**—1. Place a small drop of a solution of brilliant cresyl blue on the end of a slide and allow it to dry. Many slides can be made and kept on hand for further use.

2. Prick the finger or ear and place a drop of blood on the dye at one end of the slide.

3. Mix the blood with the dye with a match stick or glass rod and allow to stand about 30 seconds.

4. Make smear in same manner as for differential count.

5. Stain with Wright's or other suitable stain.

Slides or coverglasses may be painted with 0.5 per cent alcoholic solution of brilliant cresyl blue and dried. Blood films are then made in the usual way, using these slides or coverglasses and counterstained with Wright's stain.

## METHODS FOR COUNTING PLATELETS

**Principles.**—1. Blood platelets are stained by the polychromic dyes and are spheric or ovoid, reddish to violet, granular bodies appearing as clumps in ordinary blood films (Plate II).

2. Because of their uneven distribution in the blood, ready agglutination and tendency to disintegration, no method for counting platelets is satisfactory in every respect. They are difficult to discern because of their small size and ready attachment to particles of debris on the glassware or in the diluting fluid.

3. Generally errors tend to give low counts. However, the fragmentation of platelets or the mistaking of them for particles of stain, bacteria, portions of broken down leukocytes or erythrocytes, may give incorrectly high counts.

4. All glassware should be scrupulously clean. Diluting fluids should be fresh and filtered frequently. If capillary blood is used, puncture of a finger is preferred. The hand should be immersed in warm water and opened and closed to produce active hyperemia. The skin should be washed with soap and water and then cleansed with alcohol and ether.

5. Platelet counts are made either indirectly by determining their proportion to the erythrocytes, or directly by enumerating them in a counting chamber. The chief

objection to the latter is that it is not profitable to use the oil immersion magnification; consequently, indirect methods are preferred.

6. It is always advisable to conduct simultaneously a duplicate examination with the blood of a normal individual. Usually, only well marked reductions in the platelets below normal are of clinical importance.

**Fonio's Indirect Method.**—1. Prepare a finger and place a drop of 14 per cent aqueous solution of magnesium sulfate on the skin. Puncture through this drop.

2. With gentle pressure allow the blood to flow into the sulfate solution. When the proportion is about 1 of blood to 5 of sulfate, mix thoroughly.

3. Transfer a drop to a clean slide and make a thin smear in same manner as described for differential counts. Prepare several smears.

4. Wipe the finger clean and proceed to make a red cell count.

5. Stain the slide with Wright's blood stain, being sure that the stain is of proper reaction to stain the platelets well; otherwise they may be palely stained and difficult to count.

6. Cut a small square in a circular piece of paper and place it in the ocular of the microscope to reduce the size of the field.

7. Focus and count the number of erythrocytes and the number of platelets in the field. Continue to count fields over various parts of the slide (center and both ends) until 1000 erythrocytes have been counted.

8. The number of platelets counted to 1000 erythrocytes is multiplied by the number of thousands of erythrocytes as determined by the erythrocyte count, as per the following formula:

$$\text{Number of platelets counted} \times \frac{\text{erythrocyte count}}{1000} = \text{Platelets per c.mm. of blood}$$

Example: Patient's erythrocyte count is 4,500,000. The number of platelets counted to 1000 erythrocytes is 39. Dividing the total erythrocyte count by 1000 and multiplying by 39 gives 175,500 as the platelet count.

9. The normal by this method is from 250,000 to 500,000 per c.mm. of blood.

**Orlef's Indirect Method.**—This method <sup>15</sup> is as follows: 1. Immerse the patient's hand in warm water.

2. Wash with soap, water, alcohol and ether. Make a deep puncture in the finger without squeezing.

3. Put 5 drops of the diluting fluid in a paraffin cup prepared by melting the center of a small cube of paraffin with the heated end of a glass rod. The diluting fluid, which must be kept in a cool place and filtered every few days, is prepared as follows:

Sodium metaphosphate .....	1.0 gm.
Sodium chloride .....	0.4 gm.
Dextrose .....	0.1 gm.
Sodium bicarbonate .....	0.1 gm.
Brilliant cresyl blue .....	0.15 gm.
Distilled water .....	100.0 cc.

4. Shake the first drop of blood off the finger.

5. Place a drop of the diluting fluid on the puncture wound and turn the hand

over so that the blood will drop into the paraffin cup. Allow enough blood to fall into the cup to make a dilution approximately 1:5. This will require 1 or 2 drops.

6. Stir with a wooden applicator, the tip of which has been dipped in melted paraffin.

7. Allow to stand 1 or 2 minutes. Stir again and then, with the applicator, transfer a drop of this material to a glass slide. If several examinations are to be done, a heated glass slide placed on top of the paraffin block will keep the fluid from evaporating.

8. Place a coverglass over the drop and allow to stand for 15 minutes.

9. Examine with an oil immersion objective, counting the platelets and erythrocytes until a total of 1000 erythrocytes has been counted.

10. Make a total erythrocyte count in the usual manner.

11. Calculate as follows:

$$\text{Number of platelets} \times \frac{\text{erythrocyte count}}{1000} = \text{Platelets per c.mm. of blood}$$

12. The normal average number of platelets by this method is about 500,000 per c.mm. of blood.

**Direct Method.**—1. Prepare the *Rees-Ecker* diluting fluid (sodium citrate 3.8 gm.; formalin 0.2 cc.; brilliant cresyl blue 0.1 gm.; distilled water 100 cc.). Keep in a glass-stoppered bottle in a refrigerator. *Filter each time before use.* It should be prepared at frequent intervals as old solutions sometimes produce hemolysis by oxidation of formaldehyde liberating formic acid.

2. Prepare a finger and puncture. Draw blood to the mark 0.5 in an erythrocyte pipet and diluting fluid to 101, as for an erythrocyte count. Rapid work is necessary in order to prevent agglutination of the platelets.

3. Mix thoroughly for 2 minutes.

4. Fill a counting chamber (improved Neubauer ruling) as in making a total erythrocyte count.

5. Allow to stand about 10 to 15 minutes for the platelets to settle.

6. With the high dry objective, count all of the platelets in the *whole* finely ruled ("red cell") area. Thus a total of 25 groups of 16 squares is covered. Critical focusing is necessary for revealing the characteristic highly refractile, silvery appearing platelets. They are lilac-colored  $\frac{1}{4}$  to  $\frac{1}{2}$  the diameter of erythrocytes and usually oval, rod or comma-shaped. They may be seen singly or in groups. Great care is required in distinguishing them from artefacts and foreign material.

7. The result is multiplied by 2000 to give the number of platelets per c.mm. of blood.

## METHODS FOR DETERMINING THE COAGULATION TIME

**Principles.**—1. Prolonged coagulation time may be due to diminished (*a*) thromboplastin, (*b*) prothrombin, (*c*) fibrinogen or (*d*) the presence in the blood of an anticoagulant.

2. A determination of the coagulation time measures the clotting of blood alone, in the absence of tissue factors. Therefore, in collecting the blood it is necessary to avoid traumatization or squeezing since the exudation of tissue juice causes a shortening of the coagulation time. Consequently, methods employing venous blood are more

accurate than those employing capillary blood from a finger, because they are less likely to be mixed with tissue juices.

3. If the blood is collected in a capillary tube or test tube, it is necessary that the glassware be clean and smooth. Coagulation is more rapid the narrower the tube. Temperature conditions must be uniform since the coagulation time becomes prolonged or shortened as the temperature is lowered or raised.

4. The normal coagulation time varies with the method used; therefore, the method employed and the normal should be reported at the same time.

**Sabrazé's Capillary Tube Method.**—1. Prepare 3 capillary tubes about 8 cm. in length with a diameter of 0.8 to 1.2 mm.

2. Cleanse a finger and puncture deeply. Discard the first 2 drops of blood.

3. Secure a third drop of freely flowing blood. *Note the time.* Allow each of the tubes to fill with blood by capillary attraction.

4. Place the tubes on a table. At the end of 3 minutes, break off about 1 cm. length of tubing every 30 seconds and record the coagulating time as the interval from the time the blood appeared on the skin until a fibrin thread bridges the broken ends when they have been separated a distance of 5 mm. or more; *note the time.* The second and third tubes may be used to check the results observed with the first tube. Great care and practice are required in the breaking of the tubing so as not to break the fibrin thread.

5. The time between the appearance of the blood and the filling of the tubes and the appearance of fibrin threads is the coagulation time. The normal varies from 3 to 7 minutes.

**Slide Method.**—1. Cleanse and puncture finger as for blood count (puncture deeply to insure free flow of blood).

2. Place several drops on a clean slide (the drops should be about 4 or 5 millimeters in diameter). *Note the time.*

3. At half-minute intervals, draw a needle through one of the drops. As soon as the needle picks up fibrin threads and drags them along, coagulation has taken place. *Note the time.*

4. The time interval between placing the drop on the slide and the formation of fibrin shreds is the coagulation time. The normal time is between 2 and 8 minutes.

**Lee and White's Venous Blood Method.**—1. Rinse a small sterile syringe fitted with a sterile gauge 20 needle with sterile saline solution. Rinse an absolutely clean test tube, having an internal diameter of 8 mm., with normal saline solution.

2. Conduct a venipuncture quickly and neatly to avoid tissue juice; collect 1 cc. of blood without suction. *Note the time.* Remove the needle from the syringe and immediately run the blood into the prepared test tube.

3. If the room temperature is between 20° and 32° C., the tube may be left at room temperature; if not, stand the tube in a glass of water at 20° to 25° C.

4. Every 15 seconds tip the tube slightly until the blood no longer flows and the tube can be inverted. *Note the time.*

5. The time elapsing from the removal of blood in the syringe is the coagulation time. Normally, it varies from 5 to 8 minutes, averaging 6½ minutes. If the test tube has an internal diameter of 9 mm., the normal varies from 6 to 11 minutes.

**Howell's Venous Blood Method.**—1. Rinse a sterile all-glass syringe and needle with sterile normal saline solution. This is ejected as the syringe is held in a vertical



position so that saline solution remains in the needle and in the space between the end of the plunger and the needle.

2. Conduct a venipuncture quickly and neatly to avoid tissue juice. Remove 2 cc. of blood. Remove the needle and run the blood into a test tube with a diameter of 21 mm.

3. Keep at room temperature and from time to time observe the state of fluidity.

4. The end point is the moment when the clot is firm enough to permit inversion of the tube.

5. The normal coagulation time by this method is usually 20 to 40 minutes.

### METHOD FOR DETERMINING THE BLEEDING TIME

**Principles.**—1. The bleeding time is the time required for the blood to stop flowing from a deep cut in the finger or ear.

2. It may be prolonged with a normal coagulation time, especially in thrombocytopenic purpura. This may be due to some obscure change in the capillary endothelium, to the fact that the clot remains soft and of little value as a mechanical plug, or to defective capillary contractility.

3. Normally the bleeding time is always shorter than the coagulation time, due largely to the ability of tissue juices to promote coagulation.

**Duke's Method.**—1. If necessary, immerse the hand in warm water or rub briskly to insure adequate circulation.

2. Cleanse a finger or ear and puncture deeply so that the blood flows drop by drop without any squeezing.

3. Note the time the first drop appears.

4. Remove with filter paper each drop as it forms, care being taken not to touch the skin.

5. Note the time bleeding stops spontaneously.

6. The time interval between the appearance of the first drop and the removal of the last represents the bleeding time. The normal is 1 to 3 minutes, giving about 6 blots (Fig. 63). When the time is moderately prolonged, the twentieth blot will be about  $\frac{1}{2}$  the size of the first.

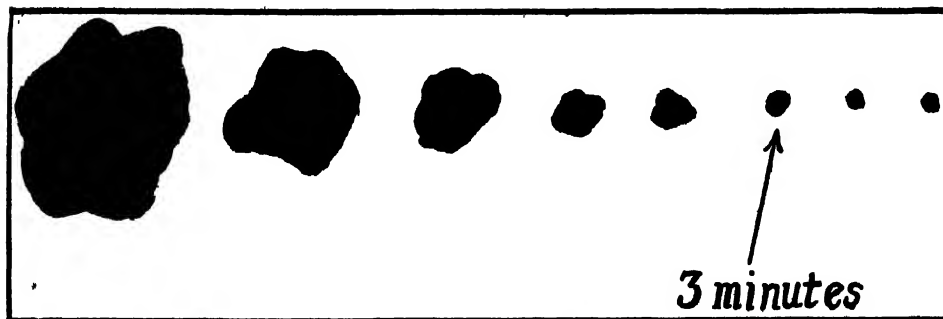


FIG. 63.—NORMAL BLEEDING TIME BY DUKE'S METHOD (NATURAL SIZE)

**METHODS FOR THE DETERMINATION OF PROTHROMBIN**

**Principles.**—1. According to Quick<sup>16</sup> 4 substances are required for the coagulation of the blood: (a) Prothrombin, (b) thromboplastin, (c) calcium and (d) fibrinogen. The first 3 interact to form thrombin which, in turn, reacts with fibrinogen to form fibrin.

2. Since the clotting time is proportional to the concentration of thrombin, one may assume that it is also proportional to the concentration of prothrombin provided thromboplastin, calcium and fibrinogen are made constant at an adequate level, for under such conditions the amount of thrombin formed is dependent upon the concentration of prothrombin present in the blood. The method consists essentially in adding to oxalated plasma an excess of thromboplastin and then recalcifying with a fixed quantity of calcium chloride. The coagulation time is an inverse measure of the concentration of prothrombin and may be evaluated by referring to a chart which was made by plotting the clotting time of known dilutions of oxalated normal blood plasma in saline and hence, presumably, of prothrombin.

**Quick's Quantitative Method.**—1. Prepare the following reagents: (a) *Sodium oxalate solution*, by dissolving 1.34 gm. anhydrous (C.P.) sodium oxalate in 100 cc. of distilled water; (b) *calcium chloride solution*, by dissolving 1.11 gm. anhydrous (C.P.) calcium chloride in 400 cc. of distilled water; (c) *thromboplastin extract*, by mixing 0.3 gm. dehydrated rabbit brain with 5 cc. of freshly prepared physiologic solution of sodium chloride containing 0.1 cc. of sodium oxalate solution. Incubate at 45° C. for 10 minutes and centrifuge at slow speed for 3 minutes to obtain a milky supernatant liquid free from coarse particles. The *rabbit brain* is prepared and dehydrated as follows: After the blood vessels have been carefully removed by stripping off the pia, the brain is macerated in a mortar under acetone, replacing the acetone several times. Dry on a suction filter and store the nonadhesive, granular powder in small ampules from which the air is evacuated for 3 minutes, by means of an oil vacuum pump, and then chilled. This powder maintains its full activity over several months. It can be obtained from various biological supply firms.

2. Place 0.5 cc. of sodium oxalate solution in a centrifuge tube. Add exactly 4.5 cc. of blood obtained by venipuncture and mix thoroughly by inverting 2 or 3 times. Centrifuge for a few minutes at moderate speed and separate the clear plasma.

3. Place 0.1 cc. of plasma in a small test tube and add 0.1 cc. of thromboplastin extract; then add 0.1 cc. of calcium chloride solution starting a stop watch at the moment of its addition. Mix gently and note the time required *in seconds* for the formation of a recognizable gel as shown by tilting the tube to a horizontal position. Conduct at 36 to 38° C. by preliminary warming in water bath.

4. Normal plasma will clot in from 12 to 13 seconds and a normal control should be included. With a decrease in prothrombin, the clotting time is delayed.

5. Determine one or more clotting times by this technic for normal plasmas. Call average value CTN. Determine one or more clotting times by this technic for patient's plasma. Call average value CTP.

$$100 \times \frac{\text{CTN} - 8.7}{\text{CTP} - 8.7} = \text{per cent of normal prothrombin time}$$

If values for CTN fall outside of 11 to 16 seconds the thromboplastic extract

cannot be considered satisfactory for use with the above equation. It may be possible to use such preparation against a curve prepared by reading the clotting time of normal plasma serially diluted with saline.

6. According to Quick,<sup>17</sup> the prothrombin concentration in the blood of normal babies is essentially the same as that of normal adult blood. Since venous blood may be difficult to obtain, the test may be conducted as follows: A drop of blood obtained by a heel or ear lobe puncture is placed on a glass slide and mixed with a drop of equal size of thromboplastin solution. The mixture is slowly stirred with a fine pointed stirring rod. By holding the slide over a light, the exact clotting time can be readily determined. Normal blood by this method will clot in 15 to 20 seconds.

**Smith Bedside Method.**—Smith and his colleagues<sup>18</sup> have recommended the following simple bedside method: 1. To prepare thromboplastin, fresh lung of ox or rabbit is ground and to each 10 grams is added 10 cc. of 0.9 per cent sodium chloride solution. Stir at intervals for several hours and filter through gauze. The extract keeps well in an ice box.

2. Place 0.1 cc. of the thromboplastin extract in a small test tube.
3. Add exactly 1.0 cc. of freshly drawn venous blood.
4. Invert the tube once to obtain complete mixing and tilt every second or two until clotting is observed.
5. Conduct a test at the same time with the blood of a normal individual.
6. The calculation is as follows:

$$\text{Clotting activity (in percentage of normal)} = \frac{\text{Clotting time of normal control}}{\text{Clotting time of patient's blood}} \times 100$$

### METHOD FOR DETERMINING HOWELL'S "PROTHROMBIN TIME"

This test is useful in the diagnosis of hemophilia. The prolonged *prothrombin time* in this condition is probably due to the failure of the platelets to disintegrate and release thromboplastic material.

1. Secure 2 cc. of blood by venous puncture in a syringe which has just been washed with physiological salt solution and without using suction.
2. Immediately place in a test tube containing 0.25 cc. of a 1 per cent solution of oxalate in physiological saline solution.
3. Mix thoroughly by inverting and centrifuge.
4. Remove the clear plasma and place 5 drops in each of four small test tubes.
5. Add 0.5 per cent calcium chloride as follows:

Tube 1 .....	2 drops
Tube 2 .....	3 drops
Tube 3 .....	4 drops
Tube 4 .....	5 drops

6. Note the time.

7. Mix gently and observe for coagulation. When a tube can be inverted without disturbing the clot, coagulation is complete. Note the coagulation time of the tube which coagulates first. This is Howell's "*prothrombin time*". It is not a measure of prothrombin concentration. Blood from a normal person should be tested at the same time as a control. The normal Howell's "*prothrombin time*" is about 10 minutes.

### METHOD FOR DETERMINING THE CALCIUM TIME

**Principles.**—This test is sometimes employed in obstructive jaundice to determine if an abnormally slow coagulation time is due to a deficiency of calcium and thus whether clotting can probably be hastened by the administration of calcium. Adding the stated amount of calcium to normal blood does not reduce the coagulation time. However, since it is doubtful that the blood calcium is ever reduced sufficiently to delay coagulation, there is little reason for the use of this test.

**Procedure.**—1. Secure 2 or 3 cc. of blood by venous puncture.

2. Place 1 cc. of blood in each of 2 test tubes having a diameter of 8 to 10 millimeters.

3. To one of the tubes add 3 drops of a 1 per cent solution of calcium chloride.

4. Observe coagulation. If the tube containing the calcium coagulates within the normal time and the tube without calcium shows delayed coagulation, the prolonged coagulation time of the blood is considered due to a deficiency in calcium.

**Lee and Vincent's Method.**—1. Rinse two 8 mm. sterile test tubes and syringe with sterile saline solution.

2. Draw blood from a vein and note the time.

3. Place 1 cc. of blood into each test tube.

4. Add 6 drops of 0.5 per cent solution of calcium chloride in saline solution to 1 tube.

5. Rotate both tubes endwise every 30 seconds until coagulation has occurred.

6. Take the time of coagulation in both tubes, counting the time from the first appearance of blood in the syringe.

7. The normal is 5 to 8 minutes in both tubes (average  $6\frac{1}{2}$  minutes).

### METHOD FOR DETERMINING THE CLOT RETRACTION TIME

**Principles.**—Retractility of a blood clot closely parallels the number of platelets. When these are numerically reduced, it is delayed, partial, or poor. Retractility is independent of the coagulation time. The blood may coagulate in normal time but retractility may be very poor. It is prolonged in thrombocytopenic purpura but normal in hemophilia.

**Procedure.**—1. Conduct venipuncture and place 3 to 5 cc. of blood in a chemically clean test tube.

2. Stopper with cotton and place in an incubator at  $37^{\circ}$  C.

3. Observe the tube at 1 hour, 18 hours and 24 hours.



FIG. 64.—BLOOD CLOT RETRACTION

A, normal or complete retractility; B, partial retractility; C, poor retractility.

4. Normally, retraction of the clot and expression of serum are appreciable after 1 hour and marked after 18 hours (Fig. 64). Occasionally, however, the clot of even normal blood fails to separate from the walls of the tube. If the clot is loosened with a platinum wire, however, retraction should occur promptly.

5. Report as poor, partial or complete.

### METHODS FOR DETERMINING THE SEDIMENTATION RATE OF ERYTHROCYTES

**Principles.**—1. The true nature or mechanism of the sedimentation of erythrocytes is not fully understood. Normally the sedimentation rate is slightly higher in women than in men.

2. Increased sedimentation is apparently due largely to changes in the plasma and especially to an increase of plasma fibrinogen or an increase of serum globulin. It bears no relationship to the total plasma proteins, the plasma albumin-globulin ratio, blood glucose, blood calcium or blood phosphorus; cholesterol, however, has an accelerating effect. The patient's temperature and exercise do not cause significant variations. After a meal the sedimentation rate is usually slightly higher than before a meal; during medication it may fluctuate to some extent. It is advisable to make the examination during a fasting state.

3. Sedimentation occurs because the density of erythrocytes is greater than the density of oxalated or citrated plasma. The most important factor affecting the phenomenon is aggregation or rouleaux formation of the erythrocytes. The larger the aggregates, the greater the speed and rate of their fall in plasma. The cause of increased rouleaux formation is not definitely known but is believed to be largely due to changes in the proteins of the plasma affecting the surface dehydration or water balance on the surface of the erythrocytes rather than to changes in the size and number of these cells in suspension.<sup>10</sup> According to some investigators, however, sedimentation tends to be more rapid than normal in marked anemia and less rapid in polycythemia. In the presence of either, a "corrected" sedimentation test may be conducted as described below.

**Technical Factors Influencing the Sedimentation Rate.**—1. The *time of day* in which blood is collected has an influence. A sedimentation rate which is slightly abnormal in the early morning may be normal in the evening, and vice versa. As a general rule, blood for the test should be collected between 10 A.M. and 4 P.M. for the most reliable results.

2. The test should be *conducted as soon as possible* after the collection of blood since a greater delay causes a decrease in the sedimentation rate.

3. The *temperature* at which the test is conducted is important since the higher the temperature, the greater the sedimentation rate. The laboratory temperature should not be below 22° or higher than 27° C. If the blood has been chilled in a refrigerator it should be warmed before the test is conducted.

4. *Anticoagulants*, with the probable exception of heparin, influence the sedimentation rate. Only when the anticoagulants recommended are used in proper concentration is this influence eliminated to an acceptable degree.

5. The *bore of the tube* does not influence the rate except that tubes less than 2 mm. in internal diameter are unsatisfactory because sedimentation is uneven in such tubes.

6. The *length of the tube* is a factor in influencing the sedimentation rate. The longer the tube, the greater the rate; however, the distance traversed is not directly proportional to the distance to be traversed, *e.g.*, the sedimentation in a tube of 300 mm. may be only slightly greater than in a 100 mm. tube at the end of 1 hour. Greater changes in the sedimentation rate occur with shorter tubes due to the influence of packing.

7. The *inclination of the tube* greatly influences the rate. Deviation of the sedimentation tube from the perpendicular position causes an acceleration of the sedimentation rate.

**Cutler Method.**—1. Aspirate 0.1 cc. of 3.8 per cent sodium citrate solution (age does not matter) into a 2 cc. sterile syringe and draw venous blood to the 1 cc. mark. Draw back the barrel of the syringe about 1 cm. and gently mix the citrate solution and blood by tilting the syringe backward and forward several times. Collection tubes may be prepared beforehand carrying 0.2 cc. of 3.8 per cent sodium citrate solution and adding exactly 2 cc. of blood followed by gentle but thorough mixing. This is convenient when several tests are to be made.

2. Set up the test within 40 hours by transferring 1 cc. of the citrated blood to a Cutler sedimentation tube of 1 cc. capacity which is graduated into 50 mm. divisions, with 0 at the 1 cc. level (Fig. 65).

3. The tube should be kept in an exactly vertical position for  $\frac{1}{2}$  to 1 hour. Readings are made every 5 minutes and recorded graphically on a chart.

**Westergren Method.**—1. Place exactly 0.5 cc. of a 3.8 per cent solution of sodium citrate in a tube bearing a mark at the 5 cc. level.

2. Withdraw 5 cc. of venous blood in a dry, sterile syringe and place exactly 4.5 cc. in the tube containing the coagulant. Invert the tube 2 or 3 times to mix the blood with the citrate solution.

3. Fill a Westergren tube (Fig. 66) to exactly the 0 mark and place it in the rack (Fig. 67). The bottom of the tube must be pressed firmly against the rubber stopper in the base of the rack before removing the finger from the top of the tube. The tube must be held firmly by the clip at the top of the rack in an exactly vertical position. Readings may be made at 5-minute

FIG. 66.—WESTER-  
GREN BLOOD SEDI-  
MENTATION TUBE



FIG. 65.—CUTLER  
BLOOD SEDIMENTA-  
TION TUBE

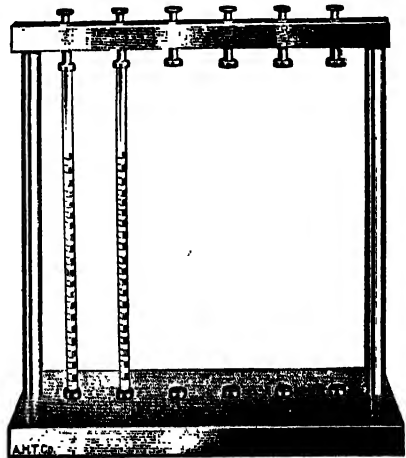


FIG. 67.—SEDIMENTATION TUBE SUPPORT  
FOR WESTER-  
GREN TUBES

intervals over a period of 1 hour and recorded graphically on a chart, or one reading may be made at the end of an hour.

**Wintrobe and Landsberg Method.**—1. Place 6 mg. of dry ammonium oxalate and 4 mg. of dry potassium oxalate in a test tube. The tubes may be prepared in advance by placing in them 0.5 cc. of a solution of 1.2 gms. ammonium oxalate and 0.8 gm. potassium oxalate in 100 cc. distilled water and evaporating the water by placing them in a hot-air oven.

2. Withdraw 5 cc. of venous blood with a dry, sterile syringe and place it in an oxalate tube. Mix gently but thoroughly with the oxalates.

3. With a capillary pipet, fill a Wintrobe hematocrit tube (Fig. 68) to the 10 cm. mark with the blood.

4. Keep the filled tube in an exactly vertical position at room temperature ( $22^{\circ}\text{C.}$  to  $27^{\circ}\text{C.}$ ) for 1 hour when the reading is made.

**Smith Micromethod.**—Smith<sup>20</sup> has described the following "micromethod" for conducting the sedimentation test in the case of infants and children in whom venipuncture may not be practical.

1. Fill the special pipet (A, Fig. 69) with a 5 per cent solution of sodium citrate and expel 0.04 cc. into the bottom of an ordinary small test tube; discard the remainder.

2. After cleansing the skin of the heel, great toe or a finger, puncture deeply enough to secure a free flow of blood.

3. Blood is then drawn into the same pipet, three successive amounts of 0.1 cc. being collected and expelled into the tube containing the citrate solution. Thorough shaking is necessary to secure adequate mixing for preventing coagulation.

4. The blood is then transferred to the special sedimentation tube (C, Fig. 69) by means of a capillary pipet (B, Fig. 69) and the test completed in the usual manner.



FIG. 68.—WINTROBE BLOOD SEDIMENTATION TUBE

**Sedimentation Readings.**—If a record is made of the erythrocyte sedimentation at frequent intervals over a period of an hour, it is possible to chart a *sedimentation curve* (Fig. 70). This procedure is recommended.

The *sedimentation rate* denotes the velocity of sedimentation per unit of time. It is a common practice to express it in millimeters at the end of an hour.

The *sedimentation time* denotes the time, in minutes, for the erythrocytes to settle to the point of packing or until sedimentation has practically ceased. The normal is always a matter of hours. It is seldom employed in routine work.

Wintrobe recommends that the uncorrected as well as the corrected sedimentation rate and the volume of packed erythrocytes be stated.

**Normals.**—The normal *sedimentation rate*, as determined at the end of an hour, depends upon the method employed and the sex of adults as follows:

(a) Cutler Method: men 0 to 8 mm.; women 0 to 10 mm.

(b) Westergren Method: men 0 to 15 mm.; women 0 to 20 mm.

(c) Wintrobe-Landsberg Method: men 0 to 6.5 mm.; women 0 to 15 mm.

**Methods for Correcting the Erythrocyte Concentration.**—As previously stated, some investigators claim that it is advisable to correct the concentration of erythrocytes before conducting the sedimentation test when they are less than 4,500,000 or more than 5,500,000 per c.mm. of blood, or the volume of packed erythrocytes is less than 45 or more than 55 per cent. Cutler does not believe this necessary in his

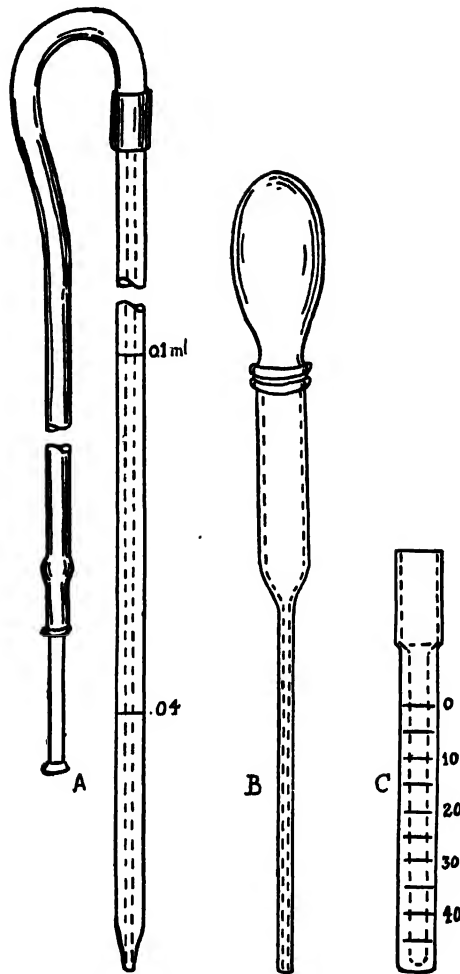


FIG. 69.—THE SMITH APPARATUS FOR DETERMINING THE SEDIMENTATION RATE IN INFANTS AND CHILDREN

*A*, pipet for measuring the citrate solution and for collecting blood; *B*, pipet for transferring citrated blood; *C*, special sedimentation tube.

method in cases of anemia. When considered advisable the concentration of erythrocytes may be adjusted as follows:

$X$  = The amount of plasma to be removed or added to obtain the desired concentration of erythrocytes by count.

$A$  = Total volume of blood in cc. to be corrected.

$B$  = Erythrocyte count of the specimen of blood in millions per c.mm.

$C$  = Desired number of erythrocytes in millions per c.mm. (4,500,000).

1. Make a total erythrocyte count of the specimen of blood.



2. If the number of erythrocytes is *less* than 4,500,000 per c.mm., centrifuge 2 cc. of the specimen at moderate speed for 10 to 15 minutes and remove the amount of plasma indicated by  $X = \frac{C - B}{C} \times A$ . Mix the balance of plasma thoroughly with the corpuscles and set up the test. Example:

Total erythrocytes per c.mm. of specimen: 3,600,000.

$$\frac{4,500,000 - 3,600,000}{4,500,000} \times 2 = 0.4 \text{ cc. plasma to be removed.}$$

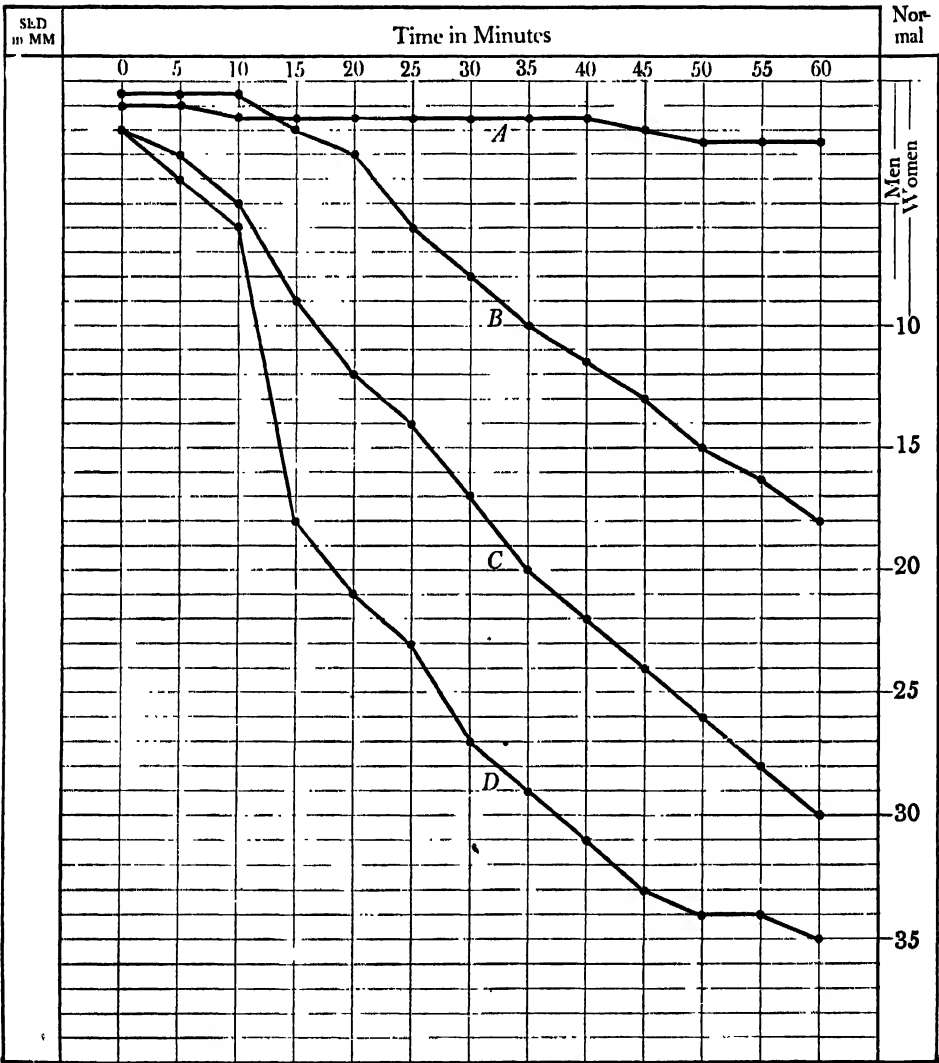


FIG. 70.—BLOOD SEDIMENTATION CHART AND GRAPHS

A, normal; B, clinically quiescent pulmonary tuberculosis; C, clinically active pulmonary tuberculosis; D, acute pyogenic infection. (From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

3. If the erythrocyte count is *more* than 5,500,000 per c.mm., centrifuge 3 cc. of the specimen and add the amount of plasma indicated by  $X = \frac{B - C}{C} \times A$  to the remaining 2 cc. of the specimen. Mix thoroughly and set up the test. Example:

$$\frac{6,500,000 - 4,500,000}{4,500,000} \times 2 = 0.44 \text{ cc. of plasma to be added to 2 cc. of specimen.}$$

4. Wintrobe and Landsberg<sup>21</sup> eliminate the necessity of correcting the erythrocyte concentration in anemia by first determining the sedimentation rate and then the cell volume by centrifuging the hematocrit tube used in their method. The rate is then corrected according to the volume of cells by referring to the chart for correction (Fig. 71). Find the horizontal line which represents the sedimentation in millimeters

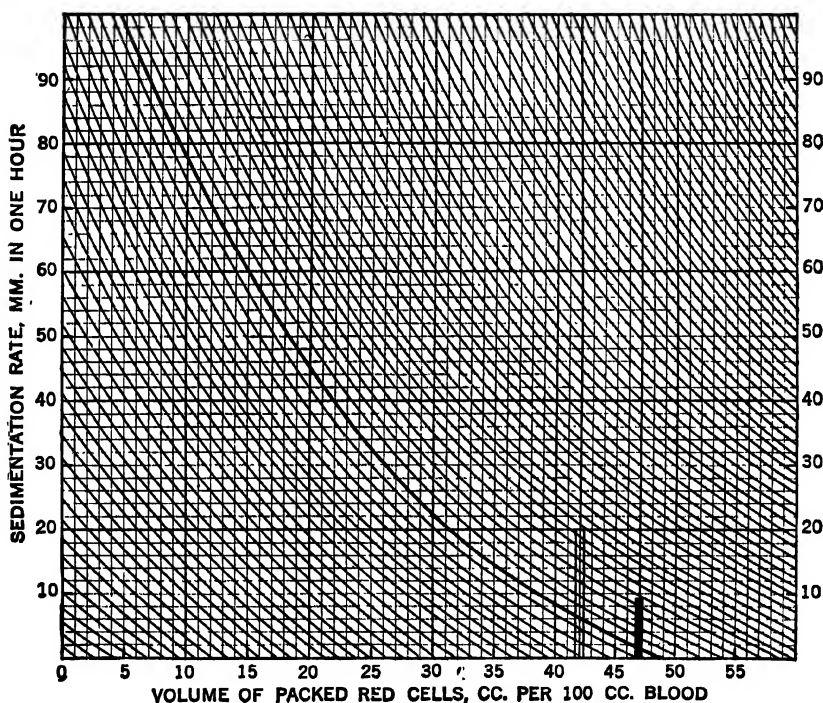


FIG. 71.—WINTROBE AND LANDSBERG'S CHART FOR CORRECTING THE SEDIMENTATION RATE FOR VARIATIONS RESULTING FROM DIFFERENCES IN THE CONCENTRATION OF ERYTHROCYTES AS MEASURED BY THE VOLUME OF PACKED CELLS

(From Wintrobe, *Clinical Hematology*, Lea and Febiger, Philadelphia.)

for 1 hour. Follow this across the chart until it intersects the vertical line which represents the blood cell volume per cent. Follow the nearest curved line until it intersects the heavy line at 42 per cent per 100 cc., if the patient is a woman, or the line at 47 per cent if the patient is a man. Then, at the point of intersection, read the value on the horizontal line for the corrected sedimentation rate. The normal average sedimentation in 1 hour by this method is 9.6 mm. for healthy women (range 0 to 20 mm.) and 0 to 9 mm. for healthy men.

### METHOD FOR CONDUCTING THE WELTMAN SERUM COAGULATION TEST

**Principles.**—1. This test is based upon the principle that normal human serum diluted with calcium chloride solution and heated in boiling water for 15 minutes will not coagulate if the concentration of calcium chloride is less than 0.04 per cent. Coagulation occurring in only the first 5 tubes is indicative of an exudative inflammatory disease ("shift to the left"); coagulation occurring in the first 7 or more tubes is indicative of healing by fibrosis ("shift to the right"). A "shift to the left", therefore, is indicative of an active inflammatory process, tissue destruction, or a state of toxemia, as in active tuberculosis or other acute infection, while a "shift to the right" is indicative of a fibrotic change in the healing of tuberculosis or some other acute infection, hepatitis, etc. The test is helpful in both diagnosis and prognosis.

2. The mechanism of the reaction is unknown.

**Procedure.**—1. From a stock 10 per cent solution of C.P. calcium chloride in distilled water prepare the following: 0.1, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02 and 0.01 per cent solutions with distilled water. These solutions are numbered from 1 to 10 beginning with the strongest concentration as No. 1.

2. Collect blood as for the Wassermann test.

3. Separate the serum and centrifuge at high speed for 10 minutes. The serum should be free of hemoglobin.

4. Place 0.05 cc. of serum into each of 10 small test tubes numbered in order from 1 to 10.

5. Add to each tube 2.5 cc. of the similarly numbered calcium chloride solution. Thus tube No. 1 receives 2.5 cc. of calcium chloride solution No. 1 (0.1 per cent).

6. Mix each tube thoroughly and place the tubes in a boiling water bath for 15 minutes. Remove the tubes and read the reaction immediately.

7. The contents of a tube may be clear, faintly opalescent, turbid, or show partial to complete coagulation. There is usually a sharp and easily noted difference between coagulation and turbidity.

8. Normal serum shows complete coagulation in the first 6 tubes and sometimes a slight or partial coagulation in the seventh tube. The normal band of coagulation is, therefore, 6 to 6½.

9. If coagulation is less than 6, it is indicative of an acute exudative process or "shift to the left"; if over 7, it is indicative of fibrosis or healing constituting a "shift to the right".

### METHOD FOR DETERMINING THE FRAGILITY OF ERYTHROCYTES

**Principles.**—1. Normally the destruction of erythrocytes goes on continually in the body.

2. In certain pathologic conditions this destruction is greatly increased, usually resulting in hemolytic anemia. Under these circumstances it is of clinical value to determine whether excessive hemolysis is due chiefly to increased fragility of the erythrocytes, as in congenital hemolytic jaundice, or to a toxic hemolytic agent in the blood causing the excessive hemolysis of erythrocytes of comparatively normal fragility.

3. Various hemolytic agents have been employed for determining the fragility of erythrocytes but hypotonic saline solutions are generally employed.

4. The fragility of erythrocytes of a normal individual should always be determined at the same time as a control. *This should not be omitted.*

5. Kracke does not recommend the use of oxalated blood for the fragility test and states that erythrocyte counts on oxalated blood from patients with hemolytic jaundice are inaccurate after about 3 hours.

**Sanford Method.**—1. Prepare a stock solution containing exactly 0.5 gm. *chemically pure* and *freshly dried* sodium chloride in 100 cc. of distilled water. Weigh on a delicate balance and measure in a volumetric flask.

2. Arrange a series of 16 small test tubes in a rack and number them 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11 and 10.

3. With a capillary pipet, place in each tube the number of drops of the stock 0.5 per cent saline solution indicated by the number on the tube. To insure equality in the size of drops the pipet must always be held at the same angle for the purpose of securing accurate results.

4. With the same pipet add to each tube the number of drops of distilled water required to bring the volume in each tube to 25 drops. Mix well. The percentage strength of the sodium chloride in any tube may then be found by multiplying its number by 0.02.

5. Obtain 1 or 1.5 cc. of the patient's blood from a vein with a small dry sterile syringe and No. 21 needle, and immediately expel 1 drop into each of the tubes. Mix each tube by inverting once or twice.

6. If the blood is not added at the bedside, collect 2 cc. with a syringe and place in a centrifuge tube carrying 0.2 cc. of a 3.8 per cent solution of sodium citrate to prevent coagulation. Mix well, centrifuge thoroughly. Discard the supernatant fluid. Add 0.7 per cent sodium chloride solution, mix well and centrifuge. Discard the supernatant saline solution. Add a volume of 0.7 per cent saline solution equal to the volume of erythrocytes which gives a 50 per cent suspension. With a syringe and No. 21 needle, add 1 drop to each tube of the test.

7. At the same time prepare a similar set of tubes with the blood of a normal individual as a control. This serves as a test of the accuracy of the saline solutions, and at the same time gives a definite standard for the interpretation of slight changes in fragility. *The results observed with the control blood should always be reported at the same time.*

8. Allow the tubes to stand 2 hours at room temperature. At the end of that time the corpuscles will have settled to the bottom and hemolysis may be recognized by the color of the supernatant fluid: Faintly pink if hemolysis is partial ("initial hemolysis"); red, with little or no sediment, if it is complete.

9. With normal blood, hemolysis usually begins in the tube containing 0.44 or 0.42 per cent saline solution (*minimal resistance*) and is complete in that containing 0.34 per cent (*maximal resistance*). When a control is used, a variation of 0.02 or 0.04 may be considered quite definite. Sanford found the average figures for minimal and maximal resistance in 23 cases of hemolytic jaundice to be 0.478 and 0.413 respectively; in chronic obstructive jaundice, 0.396 and 0.31. In secondary and pernicious anemia the figures vary only slightly from the normal, with a tendency to slight increase of resistance. In purpura resistance is normal.

### METHOD FOR THE EXAMINATION OF STERNAL BONE MARROW

**Principles.**—1. Examinations of the bone marrow during life are not infrequently of considerable clinical value in the diagnosis of diseases of the blood and of the blood-forming organs. This is especially true when the blood findings are atypical or inconclusive, as in differentiating "aleukemic" leukemia from agranulocytosis, purpura haemorrhagica and aplastic anemia.

2. However, the examination of bone marrow requires unusual skill and experience. This is due to the difficulty so frequently encountered in the recognition of the many different kinds of cells encountered belonging to the granulocytic, lymphocytic and erythrocytic series, since the marrow consists of a variety of blood cells and their precursors, as well as fat cells, blood vessels and a framework of reticulum. During the first few years of infancy and childhood practically all of the marrow is of the red variety and highly cellular. Fat cells begin to appear between the ages of 5 and 7 years. At about maturity, the actively hematopoietic marrow is found only in the sternum, ribs, vertebrae, bones of the skull, the innominate bone and, to some extent, in the proximal epiphyses of the femur and humerus. The total bone marrow in adults has been calculated to vary from 1600 to 3700 gm. with only about half in an active state.

3. Because of rapid autolysis, marrow examinations conducted postmortem are frequently unsatisfactory unless removed within 2 hours after death. Specimens should be taken from several bones but if only one is to be taken, Custer<sup>22</sup> recommends the middle of the femur because the marrow is more labile than that of the tibia or even that of the ribs.

**Technic of Sternal Biopsy.**—From the standpoint of clinical diagnosis, examinations of the marrow during life are of most interest and value, especially since 1923 when the method of *sternal trephining* was introduced by Seyfath. This method is generally favored because it yields material which may be used for preparing smears, touch preparations, wet preparations for supravital staining, and especially blocks for the preparation of sections. As compared with *sternal puncture*, however, it has the disadvantage of not being as readily repeated at short intervals.

The technic of the latter, introduced by Arinkin<sup>23</sup> in 1929, is much more simple. The upper portion of the bone, between the second and third ribs, is the site of choice because it is less likely to bend or give at that point. Furthermore, marrow is present in that area even in infants of 1 to 2 years of age. The manubrium is more likely to contain fat and the lower third of the sternum is unsatisfactory because congenital abnormalities are common in that region.

1. The skin is prepared as for sternal trephining and the whole operation conducted with scrupulous aseptic precautions. A cushion may be placed beneath the shoulders. The skin, deeper tissues and periosteum are infiltrated with a sterile 1 per cent solution of procaine. A short-beveled, 18-gauge needle with a lumen of 1 to 2 mm. is employed. It should be short to avoid bending and preferably provided with an adjustable guard, the Sharp needle (A. S. Aloe Company, St. Louis, Mo.) being recommended. The guard is set at 1 cm. if the patient is an adult, or at 0.6 to 0.2 cm. if a child; the outer lamina of the sternum varies considerably in thickness, ranging from 0.2 to about 5 mm. The needle is pushed vertically with a slight rotating or boring motion into the sternum in the midline. A "give" is felt as the marrow cavity is

entered. The needle is then passed about 1 or 2 mm. farther in as the cavity is normally 5 to 15 mm. in depth.

2. The stylet is then removed and a sterile 2 or 5 cc. tight-fitting syringe is attached with the aspiration of 1 or 2 cc. of liquid or semi-liquid marrow without too much suction to avoid pain. If no marrow is obtained, the stylet may be replaced and the marrow penetrated more deeply. If this fails, 0.5 cc. of sterile saline solution or the patient's own plasma (prepared beforehand and in readiness) may be injected to aid the dislodgment of cells and then removed. Upon removal of the needle the puncture wound is sealed with collodion and cotton.

3. The material is then sent to the laboratory for examination of smears, dry imprints, supravital preparations, wet preparations and possibly of sections. Smears stained by the Wright or May-Grünwald-Giemsa stains are of particular value in a study of the cells. Smears may be stained also by the peroxidase method. If Wright's stain is used it may be advisable to dilute it with an equal quantity of absolute methyl alcohol. This is allowed to remain on the smear for 2 minutes when water is added and the mixture allowed to stain for 4 to 8 minutes.

4. When properly conducted the method is safe, causes but little discomfort and may be classed with such routine examinations as spinal puncture and pleural tap. Hemophilia may be the only contraindication on account of the danger of bleeding. The most important criticism is that only a small and not necessarily representative sample of marrow is obtained with the chances of missing the lesions which are patching in character, as in Gaucher's disease, multiple myeloma, etc.

**Normal Bone Marrow.**—Erythrocyte counts and hemoglobin estimations may be made. The results are about the same as those of blood or slightly lower. The leukocytes range from 10,000 to 190,000 per c.mm. Differential counts are of most value (Fig. 72). At least 500 to 1000 cells should be examined. Even under these circumstances, considerable variations may be found not only between several preparations of the same material, but especially between specimens removed at different times. The relative percentages of nucleated cells for adults are approximately as follows: <sup>24</sup>

<i>Cells</i>	<i>Range</i>	<i>Average</i>
Myeloblasts .....	0.3 to 5.0 .....	2.0
Promyelocytes .....	1.0 to 8.0 .....	5.0
Myelocytes: Neutrophilic .....	5.0 to 19.0 .....	12.0
Eosinophilic .....	0.5 to 3.0 .....	1.5
Basophilic .....	0.0 to 0.5 .....	0.3
Metamyelocytes .....	13.0 to 32.0 .....	22.0
Polymorphonuclear neutrophils .....	7.0 to 30.0 .....	20.0
Polymorphonuclear eosinophils .....	0.5 to 4.0 .....	2.0
Polymorphonuclear basophils .....	0.0 to 0.7 .....	0.2
Lymphocytes .....	3.0 to 17.0 .....	10.0
Plasma cells .....	0.0 to 2.0 .....	0.4
Monocytes .....	0.5 to 5.0 .....	2.0
Reticulum cells .....	0.2 to 2.0 .....	0.2
Megakaryocytes .....	0.03 to 3.0 .....	0.4
Macroblasts .....	1.0 to 8.0 .....	4.0
Normoblasts .....	7.0 to 32.0 .....	18.0

In children the leukocyte:nucleated erythrocyte cell ratio is about 8:1, or even 2:1, while in adults it is normally 3 or 4:1. In young children there are relatively more immature neutrophilic cells and more lymphocytes than in adults.

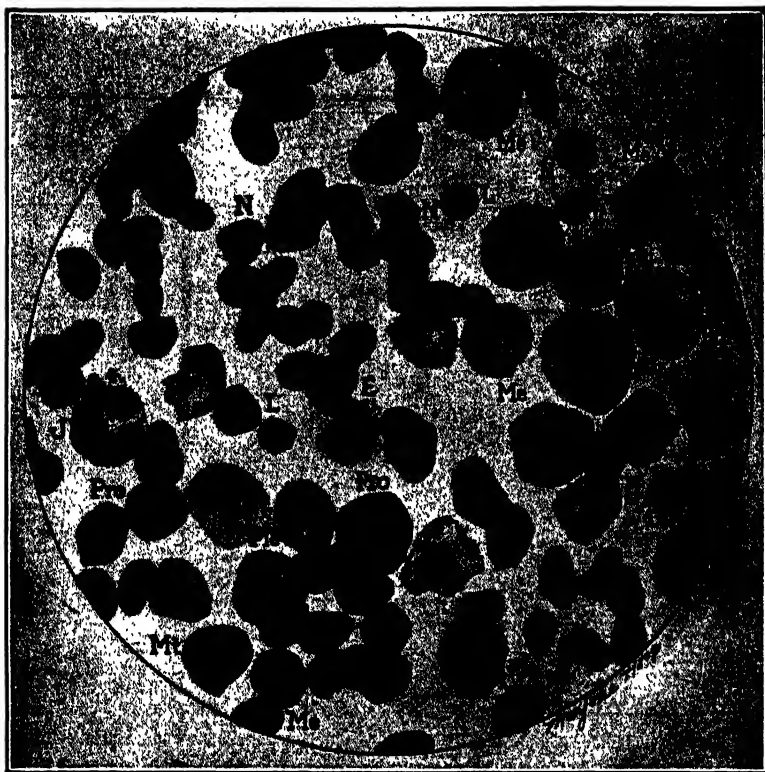


FIG. 72.—SMEAR OF BONE MARROW AS OBTAINED BY STERNAL PUNCTURE

There is a well marked "shift to the left" with a preponderance of myelocytes at the expense of metamyelocytes and segmented forms. *Mt*, myeloblast; *Me*, myelocyte (differentiated); *J*, juvenile or metamyelocyte; *E*, eosinophil; *L*, lymphocyte; *P*, plasma cell; *N*, normoblast. (From Wintrobe, *Clinical Hematology*, Lea and Febiger, Philadelphia.)

**Bone Marrow in Disease.**—In many diseases gross as well as microscopic changes may occur in the bone marrow. For example, its unusual red color in pernicious and other severe anemias with the displacement of fat or displacement of the latter by gelatinous albuminoid material as in starvation states and wasting diseases.

However, of special clinical importance is the information sometimes obtainable from a skilful microscopic examination in differential diagnosis. This is particularly true in those diseases of the hemopoietic system in which no definite changes in the blood are found. Sternal biopsies have frequently demonstrated the presence of "aleukemic" leukemia with the ruling out of aplastic anemia or Banti's disease. Furthermore, they are frequently helpful in distinguishing between leukemoid conditions and true leukemia, pseudopernicious anemia from true pernicious anemia, granulocytopenia due to marrow aplasia from that due to drug allergy, as well as frequently revealing primary or secondary bone tumors. The important changes found in many of these diseases are summarized in Table 9.

**TABLE 9.—BONE MARROW EXAMINATIONS IN DISEASE**

Disease	Important Changes
Pernicious and related anemias	Untreated or during relapse: (a) Increase of nucleated erythrocytes; (b) preponderance of megaloblasts; (c) increase of reticulum (Ferrata) cells; (d) abnormal leukopoiesis, especially lymphocytes; (e) reduction in megakaryocytes.
Aplastic anemia	Chiefly red blood corpuscles. Relative lymphocytosis constituting 60 to 100 per cent of the nucleated cells. Striking immaturity of the red and white corpuscles. May be normally cellular or hyperplastic.
Acute hemolytic anemias	Markedly hyperplastic; 60 per cent or more of the nucleated cells belong to the erythrocytic series; leukocytes relatively reduced.
Chronic hemolytic anemias	Normoblastic hyperplasia characteristic (normoblasts or macroblasts); no megaloblasts.
Sickle cell anemia	Largely nucleated red cells (chiefly normoblasts). May be moderate "shift to the left" of the myeloid leukocytes; eosinophils relatively increased; megakaryocytes may be increased.
Hypochromic microcytic anemia	Hyperplastic; increase of normoblasts; no megaloblasts; granulopoiesis usually normal.
Congenital hemolytic jaundice	Erythropoietic hyperplasia of the normoblastic type; no megaloblasts or giant abnormal leukocytes.
Purpura haemorrhagica	Many megakaryocytes; usually an increase of erythroid elements due to severe hemorrhage and anemia.
Polycythemia vera	Dark red and very cellular; hyperplasia of all elements; moderate increase of nucleated erythrocytes; sometimes an increase of megakaryocytes, myelocytes and myeloblasts.
Myeloblastic and myelocytic leukemia	Marked hyperplasia; crowded with myeloblasts and more primitive cells. In eosinophilic leukemia preponderance of eosinophils. In monocytic leukemia myelocytes and myeloblasts; also "monoblasts" and "monocytes" in some cases. In chronic myelocytic leukemia the differential count is similar to that of the blood.
Lymphocytic leukemia	May be only slightly changed; usually, however, a well marked lymphocytosis (30 to 90 per cent of the cells).
"Aleukemic" leukemia	Of great value in diagnosis. Frequently the changes are identical with those with typical blood findings. Sometimes misleading when only a few cells are obtained. But pronounced immaturity of the leukocytes may be observed when cellularity is reduced. In leukopenic cases of lymphocytic leukemia, marrow lymphocytosis may be slight or absent.
Infectious mononucleosis	Increase of lymphocytes or moderate shift to left of myeloid leukocytes. Chiefly of value from a negative standpoint in the sense that findings characteristic of leukemia are absent.



**TABLE 9.—BONE MARROW EXAMINATIONS IN DISEASE (Contd.)**

Agranulocytosis	Normal erythropoietic tissue and normal numbers of megakaryocytes. Striking lack of granulocytes. Plasma cells, lymphocytes and reticulum cells may be increased.
Hodgkin's disease	Findings variable and nonspecific. May be slight shift to left in the myeloid cells; also slight monocytosis or moderate eosinophilia. No lymphocytosis. Relative reduction in nucleated red cells.
Multiple myeloma	Various types described as myeloblastic, lymphoblastic, erythroblastic, etc., with "plasma cells" the usual designation. <i>Myeloma cells</i> most characteristic constituting 3 to 65 per cent of all cells present.

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## METHODS FOR THE EXAMINATION OF URINE

**Normal Urine.**—It is not possible to accurately define a normal urine, even if the type and amount of diet and the fluid-intake are known, because variations may occur according to external and internal temperatures, physical activity, physiologic variations in metabolism and the composition of plasma reaching the kidneys, but the average physical, chemical and microscopical composition of the urine of normal individuals may be summarized as shown in Table 10.

**TABLE 10.—AVERAGE NORMAL URINE**

Formation	Glomerular filtration followed by selective tubular re-absorption. Tubular excretion and secretion doubtful.
Amount	Adults: 1000 to 1600 cc. per 24 hours; children 3 to 4 times as much as adults per kilogram of body weight. During the day 2 to 4 times as much voided as during the night.
Turbidity	Normally clear when freshly voided but may be cloudy due to phosphates. All urine becomes cloudy or turbid upon standing. Alimentary lipuria may occur (opalescence); also from accidental contamination with oils.
Color	Yellow-amber due to normal pigments.
Odor	Normally aromatic due to volatile acids; especially marked in concentrated urine. Ammoniacal and "urinous" upon decomposition. Peculiar odors may be due to various articles of diet and drugs.
Reaction	Acid, due to acid phosphates and traces of organic acids. Average 24-hour urine requires from 200 to 500 cc. of decinormal NaOH to neutralize the acid. Occasionally alkaline due to alkaline salts. All urine without preservatives becomes alkaline upon standing.
Specific gravity	Varies according to solids in solution from 1.010 to 1.030 averaging 1.015 to 1.025. Of little value with random specimens; best determined with a sample of total 24-hour urine.
Total solids	Related to specific gravity under normal conditions. Accurate chemical methods preferred for estimation. Varies from 60 to 70 grams in 24-hour urine. Decreases after 45 years of age.
Albumin and Globulins	About 0.075 gm. in 24-hour urine and too small in amount for detection by ordinary qualitative and quantitative tests. Physiologic albuminuria may occur from excessive proteins in diet.
Bence-Jones Protein	Contained in normal bone marrow. Normally absent in the urine.
Proteoses	Absent.
Mucin	Traces may be present.
Nucleoprotein	Absent.

**TABLE 10.—AVERAGE NORMAL URINE (Contd.)**

Urea	Principal waste product of metabolism; 20 to 30 gm. in total 24-hour urine on average diet. Estimations based on random samples of urine are of little or no value. Urea nitrogen 9 to 14 gms. for 24-hour urine.
Ammonia	In terms of $\text{NH}_3$ about 0.6 gm. per 1000 cc. Free ammonia produced from decomposition of urea after voiding or in cystitis with retention.
Total nitrogen	On a mixed diet 12 to 18 gms. per 24-hour urine. Varies directly with the protein ingested.
Uric acid	0.4 to 1 gm. per 24-hour urine; increased by exercise and foods rich in the purines. Excreted as urates and in the free state.
Creatine	In adult males 0 to 196 mg. per 24-hour urine; more commonly found in the urine of women; regularly in children (10 to 15 mg. daily).
Creatinine	About 1.25 gm. per 24-hour urine. Constant and independent of diet.
Amino acids	Total of free and combined 0.5 to 1.0 gm. in 24-hour urine.
Hippuric acid	About 0.7 gm. per day.
Guanidine	Equivalent to an excretion of 3 to 10 mg. of guanidine base per 24-hour urine.
Glucose	Reducing substances normally present (glycuresis) including trace of glucose; reduction of Fehling's and Benedict's reagents may also occur by uric acid, nucleoprotein, conjugate glucuronates and chloroform.
Pentose	Traces usually as the optically inactive form of arabinose.
Levulose	Usually absent.
Lactose	Minute traces may occur.
Lactic acid	0.05 to 0.2 gm. per 24-hour urine. Increased by severe exercise.
Galactose	Usually absent.
Acetone	Minute traces may be present, especially in the urine of young children. Should always be tested for routinely in preoperative surgical cases.
Diacetic acid	Absent.
Betahydroxybutyric acid	Absent.

**TABLE 10.—AVERAGE NORMAL URINE (Contd.)**

Bile and biliary pigments	No bilirubin. Trace urobilin. Urobilinogen (1 to 4 mg. per 24-hour urine) reacting in dilutions up to 1:20 of urine. No salts of biliary acids.
Diazo substance	Absent.
Indican	4 to 20 mg. per 24-hour urine. Increased by meat diet.
Chlorides	Largely sodium chloride; 10 to 16 gm. in 24-hour urine. Influenced by diet.
Phosphates	2.5 to 3.5 gm. in 24-hour urine; 2/3 as alkaline phosphates and 1/3 as earthy phosphates.
Calcium	0.06 to 0.2 gm. per 24-hour urine (0.1 to 0.4 gm. calculated as CaO). The amount is dependent upon the calcium content of the diet.
Drugs and Poisons	Lead 0.010 to 0.100 mg. per liter; arsenic 0 to 0.15 mg. per 24-hour urine; mercury none unless exposed to it.
Casts	Hyaline casts occur averaging about 1040 in 12-hour concentrated night urine (Addis). By ordinary methods of examination usually absent except occasional hyaline casts in the urine of elderly individuals.
Leukocytes and Pus	Few present; increased by contamination with vaginal discharges.
Erythrocytes	Vary from none to 425,000 (average 65,750) in 12-hour night urine (Addis). When containing 70,000 to 100,000 the urinary sediment may show 4 to 10 per high power field which may be normal. An occasional erythrocyte, therefore, is within normal. Increased through contamination by uterine hemorrhage (menstruation, etc.); also by trauma due to catheterization.
Epithelium	Few cells always present. Three main varieties: (a) Small round; (b) transitional and (c) squamous.
Mucus	Traces.
Spermatozoa	Usually absent but may occur after nocturnal emissions; prolonged continence and after coitus in both sexes.
Tissue fragments	Absent.
Crystals	Commonly: Uric acid and urates; calcium oxalate; phosphates; calcium carbonate; ammonium biurate. Rarely: Calcium sulfate; cholesterol; hippuric acid.

## COLLECTION

1. Single specimens collected at varying times in the day may yield different results, especially in amounts of glucose and albumin; this is a reason for the varying reports frequently obtained from different laboratories examining specimens of urine of the same person collected at different times. Specimens voided 2 or 3 hours after a meal are likely to contain the largest amounts of glucose or albumin; those passed first in the morning are least likely to contain them.

2. If any dependable data are desired regarding the quantitative composition of the urine, the examination of the mixed excretion for 24 hours is generally necessary. *No quantitative test can be of much clinical value unless a sample of the mixed 24-hour urine is used.* In collecting the urine the bladder may be emptied at any given hour, e.g., 8 A.M., the urine discarded and all the urine voided from that hour up to and including that passed the next day at 8 A.M. saved, thoroughly mixed, accurately measured, and 4 to 8 ounces taken for analysis.

3. In certain pathological conditions it is desirable to collect both day and night specimens. Urine voided between 8 A.M. and 8 P.M. may be taken as the day sample and that voided between 8 P.M. and 8 A.M. as the night sample.

4. Containers used for collection of urine should be *chemically clean*. Careful cleansing is required, particularly in hospital laboratories, to avoid the possibility of carrying over traces of albumin and sugar. Traces of syrup in insufficiently washed medicine bottles are sometimes responsible for errors.

5. Contamination with vaginal discharges may account for the presence of albumin and pus; contamination with menstrual discharges may account for the presence of albumin and blood. Both should be carefully avoided, as well as contamination with feces.

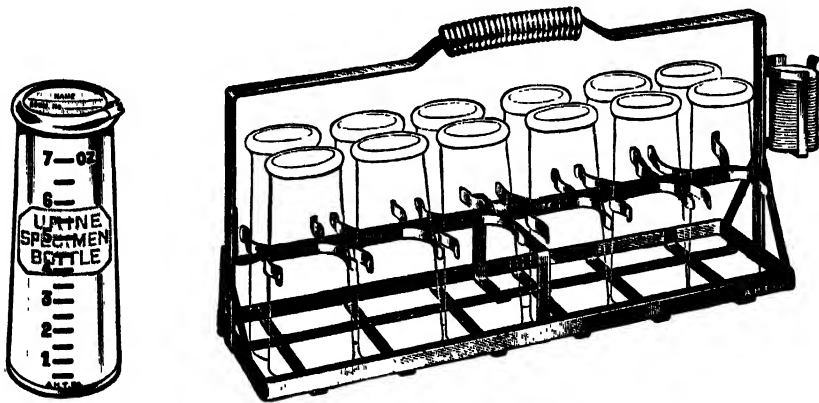


FIG. 73.—URINE SPECIMEN BOTTLE AND PORTABLE RACK

6. Urine to be examined for tubercle bacilli may be voided although there are chances of contamination with smegma bacilli. Urine for other bacteriological examinations should be collected aseptically, by sterile catheter, into sterile containers (*without a preservative*), as it is almost impossible otherwise to avoid bacterial contamination, especially with *B. coli* and staphylococci.

7. In male infants urine may be secured by applying a condom over the penis; or a small wide-necked bottle may be held in place by bandages or adhesive strips. In female infants the neck of the bottle may be passed through an oblong piece of adhesive plaster, firmly attached there in a way to prevent leakage, and the plaster then applied over the vulva and perineum; or a glass cup, such as is used to hold feed in bird cages, may be kept in place over the vulva by the pressure of the diaper. Another procedure is to permit the infant to lie upon a small, circular, rubber aircushion with an opening in the center and a small basin placed beneath the opening.

8. In hospital laboratories special containers and portable racks may be employed (Figs. 73 and 74).

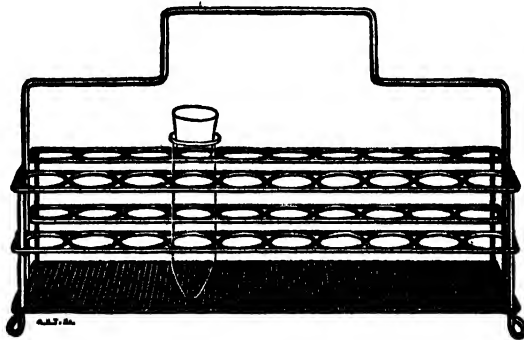


FIG. 74.—THE BOERNER URINE RACK

## PRESERVATION

Decomposition sets in rapidly, especially in warm weather, and interferes greatly with all examinations. An ideal preservative should prevent the growth of bacteria and molds; should not interfere with the accuracy of physical, chemical and microscopical examinations; should be readily soluble, of low cost, and preferably a solid.

1. If a *refrigerator* is available, samples may be kept in it until examined. Samples for pregnancy tests must be so preserved. Avoid freezing.

2. A small piece of *camphor*, sufficient to give a saturated solution, may be used.

3. *Thymol*, if used, should not exceed 0.1 gram per 100 cc. of urine. An excess may interfere with albumin determinations. It is not as good as formerly surmized and is unsatisfactory when urine contains sugar, acetone or diacetic acid; also when urine is to be examined for phenol and quantitatively for phosphates or magnesium.

4. *Formalin*, in proportion of 1 or 2 drops to the ounce, is the most satisfactory of all, especially for the preservation of the formed elements. An excess may interfere with tests for indican, albumin and sugar, and produce a precipitate.

5. *Boric acid*, 5 grains for each 4 ounces, delays decomposition but may interfere with sugar determination and precipitate rhombic crystals of uric acid.

6. *Toluol* may be used, especially for specimens to be examined for acetone and diacetic acid. Simply add enough to form a thin layer on the surface. It is a very satisfactory preservative for routine use.

7. *Chloroform* is the least satisfactory and should not be used as it interferes with sugar determinations and microscopical examinations.

### PHYSICAL EXAMINATION

**Amount.**—1. The amount of urine in 24 hours varies with the fluid intake and the quantity of water eliminated by the skin, lungs and bowels. Profuse sweating and diarrhea reduce the output. The volume is less in the standing position than in recumbency. The output is greater upon a high than upon a low protein diet. The urine of the day is normally 2 to 4 times greater in amount than that excreted during the night, even though the total fluid intake is the same for both periods.

2. Young children excrete from 3 to 4 times more urine than adults according to body weight. Women normally excrete less than men.

3. The normal volume of urine excreted in 24 hours varies according to age as follows:

1 and 2 days .....	15 to 60 cc.
3 to 10 days .....	100 to 300 cc.
10 days to 2 months.....	250 to 450 cc.
2 months to 1 year.....	400 to 500 cc.
1 to 3 years .....	500 to 600 cc.
4 to 8 years .....	600 to 1000 cc.
9 to 15 years .....	800 to 1400 cc.
over 15 years .....	1000 to 1600 cc.

4. **Polyuria** designates an abnormal increase in volume per 24 hours; *oliguria* an abnormal decrease; *anuria* a total cessation of urinary excretion although the term is sometimes erroneously used for failure to pass urine because of complete ureteral or urethral obstruction; *nocturia*, for an increase of night urine above 500 cc. in adults although the term is frequently and erroneously used for increased frequency of urination during the night without an actual abnormal increase of the total urinary output above normal.

**Color.**—1. Normally, the urine is yellow or reddish-yellow (amber), due to the presence of such pigments as urochrome, urobilin, uro-erythrin and porphyrins. Dilute urine (polyuria) of low specific gravity is usually pale yellow or greenish-yellow, while concentrated or scanty urine (oliguria) is dark amber. Acid urine is usually darker than alkaline urine.

2. Color may be greatly changed by the presence of blood, melanin and other abnormal pigments, various drugs, poisons, etc.:

Erythrocytes: red to brownish red; smoky

Hemoglobin: red to reddish-brown or black

Hematoporphyrin and other porphyrins: port wine

Neoprontosil: dark-red

Pyridium: orange-red

Santonin: golden-yellow

Acriflavin: yellowish-green

Methylene blue or thymol: greenish-blue

Bilirubin and biliverdin: yellowish-green

Urobilin in excess: dark brown

Hemogentisic acid and other alkaptons: brown, turning black

Hydroquinon or pyrocatechin: dark brown to black; smoky

Chyle or marked contamination with petrolatum or milk: milky

3. Vogel's color scale is useful, the urine being filtered and viewed by transmitted light in a glass 3 or 4 inches in diameter: Pale yellow, light yellow, yellow, reddish-yellow, yellowish-red, red, brownish-red, reddish-brown and brownish-black. To these may be added greenish-yellow, olive, milky, etc.

**Odor.**—1. Normal fresh urine has a characteristic aromatic odor due to volatile acids or a substance called "urinod". It is more marked in concentrated urines.

2. Abnormal odors may be due to foods, like asparagus, drugs like oil of turpentine, menthol, cubebs, copaiba, sandal-wood oil, etc. Other abnormal odors are as follows:

Ammoniacal: due to decomposition of proteins

Fruity or sweetish: probably due to acetone

Putrid: due to hydrogen sulfide from the decomposition of pus  
or in cystinuria

Fecal: due to contamination with feces or *B. coli*

**Transparency and Sediments.**—1. Freshly passed urine is usually clear or transparent but may be cloudy due to the presence of phosphates or pus. The former disappears upon the addition of acid; the latter does not, but may become gelatinous (Donné's test). A freshly passed urine may also be cloudy with bacteria, or comparatively clear with numerous shreds of mucopurulent material and especially in chronic urethritis.

2. A record of the transparency is only of value in comparatively fresh specimens. Upon standing all become cloudy with bacteria and alkaline salts as the result of decomposition.

3. Upon cooling and standing, all specimens develop a faint cloud of mucus, leukocytes and epithelial cells which settle to the bottom—the so-called "nubecula". This has no significance.

4. Acid urines may develop a white or pinkish sediment of amorphous urates.

5. Alkaline urines may develop a heavy white sediment of amorphous phosphates.

6. Pus gives a heavy mucoid whitish sediment.

7. Blood gives a reddish-brown smoky sediment.

8. Bacteria give a uniform cloudiness which cannot be removed by ordinary paper filtration. Such urines can usually be rendered comparatively clear by adding a small amount of purified talc or infusorial earth, shaking well and filtering.

9. The following terminology is recommended:

(a) Clear, slightly cloudy, cloudy, very cloudy.

(b) Sediment: Slight, moderate or heavy; white, pinkish, red,  
brown, reddish-brown, etc.; shreds present or absent.

## METHODS FOR DETERMINATION OF REACTION

**Principles.**—1. Normally, the mixed 24-hour specimen is slightly acid in reaction, due largely to the monobasic (chiefly monosodium) salts of phosphoric acid plus small amounts of free organic acids (uric, lactic and hippuric). As a result, the usual total titratable acidity of 24-hour urine is between 200 and 400 cc. of N/10 standard acid but, in health, may vary from 100 to as much as 600 cc. In terms of pH the average normal is about 6.0 but the range in health may vary from as low as 4.6



to as high as 8.0.<sup>5</sup> No single test may be accepted as giving the range for any individual. Indeed, normality is characterized by variability.

2. Water excretion, emotional status, exercise, fatigue, meals and rate of pulmonary ventilation are all factors affecting urinary reaction.

3. As shown by Leathes, the urine passed a short time after rising in the morning may be less acid than that found during sleep (*morning alkaline tide*) which he ascribed to depression of the respiratory center during sleep with the retention of carbon dioxide. An alkaline tide also occurs within an hour after a meal (*postprandial alkaline tide*) and sometimes freshly voided urine is both cloudy and highly alkaline due to phosphates (phosphaturia).

4. The acidity of the urine is normally influenced by diet, fluid intake and also by various drugs. Cereals, meat and fish tend to increase it while most fruits (except plums, prunes and cranberries) tend to reduce it because of basic radicles (alkaline ash) with their rejection by the tubules. A high protein diet, however, increases acidity because of a production of an excess of sulfuric and phosphoric acids which are eliminated by tubular rejection. Acidity is also increased by a diet sufficiently high in fats to produce ketosis; also by an acid-ash diet which is more effective in the long run. Fasting and starvation, in which the body proteins are metabolized, also tend to increase the titratable acidity.

5. The administration of large amounts of hydrochloric, phosphoric and mandelic acids, as well as of ammonium chloride, ammonium nitrate, ammonium mandelate, and calcium chloride, tend to increase the acidity of the urine. The maximum acidity attainable, however, is about pH 4.6.

6. Alkaline urines are frequently observed in cystitis and pyelonephritis because of the formation of ammonia from urea by bacteria.

7. Unless properly preserved, urine on standing, ultimately becomes alkaline because of the formation of ammonia by contaminating bacteria.

**Litmus Method.**—For ordinary purposes the reaction may be determined with good grades of blue and red litmus papers:

Blue turning red: acid

Red turning blue: alkaline

No change in either: neutral

Changes both red and blue: amphoteric

**Total Acidity Method.**—The total acidity may be determined by titration according to the method of Folin and Wu as follows:

1. Use a sample of mixed 24-hour urine, as fresh as possible, and accurately measured.

2. Place 25 cc. in a small flask or evaporating dish. Add 2 drops of 0.5 per cent alcoholic solution of phenolphthalein and 15 grams of neutral finely pulverized potassium oxalate.

3. Shake vigorously for 2 minutes.

4. Immediately titrate with N/10 sodium hydroxide solution, shaking after each addition, until the first permanent pink color appears.

5. Read off amount of N/10 sodium hydroxide used.

6. Multiply by 4 to estimate amount required for 100 cc. of urine and report accordingly (normally 25 to 40 cc.).

7. Calculate and report amount required for total 24-hour specimen. Normally, 200 to 400 cc., but in health may vary from 100 to as much as 600 cc., largely according to diet.

**Hydrogen Ion Concentration Method.**—There are numerous methods for determining the hydrogen ion concentration (or pH) of the urine but the simplest method, satisfactory for clinical purposes, is that involving the use of nitrazine paper (phenaphthazine) as follows:

1. With a clean glass rod, transfer a drop of urine to the surface of a strip of nitrazine paper (Squibb) and spread evenly by stroking or leave a small drop on the paper. After 1 minute, compare with the color chart furnished. The paper may be dipped into the urine three consecutive times and the excess shaken off. Compare after 1 minute.

2. The color comparison chart reads from pH 4.5 to 7.5 in 0.5 divisions. It is possible to interpolate between these divisions by estimating the color half-way between them.

3. The average normal is about pH 6.0 but the range in health may vary from as low as 4.6 to as high as 8.0.

## METHODS FOR DETERMINATION OF SPECIFIC GRAVITY

**Principles.**—1. The normal specific gravity of the urine varies directly according to the amounts of solids in solution (chiefly chlorides and urea) and inversely according to volume. Since the former fluctuates according to diet, and the latter according to fluid intake versus fluid loss through the skin, lungs and bowels, the range of normal may be anywhere between 1.010 to 1.030, with a general average between 1.015 and 1.025.

2. The first voided urine of the morning is generally more concentrated and of higher specific gravity than that passed during the day; a high fluid intake may readily reduce it below 1.010 while a low fluid intake or much fluid loss through the skin and lungs, by exercise or from the bowels after laxatives, may raise it to 1.030 or higher. Under the conditions, *the specific gravity of a single random specimen of urine in health may readily enough fall within the upper and lower limits of the urine in disease.*

3. In disease, the specific gravity of a mixed 24-hour specimen of urine may vary from as low as 1.001 to as high as 1.060. The relationship between specific gravity and volume may be lost. Thus, while the fluid intake may be normal or rigidly restricted, a kidney, the function of which is seriously impaired, may be unable to concentrate the urine sufficiently to raise the specific gravity to 1.010; on the other hand, when the fluid intake is high, the excretion of a urine of low specific gravity (a feat readily accomplished by the normal kidney) is beyond the capacity of a seriously diseased one.

4. If the specimen contains but a small or average amount of sediment it makes but little or no difference whether the urine is mixed up or the specific gravity taken without mixing in order to use the sediment later for microscopical examination. If, however, there is a large amount of sediment the specific gravity is almost always increased by about 0.002 after thorough mixing and under these circumstances unmixed urine is preferred.

**Procedures.**—For ordinary determinations the Squibb urinometer (Fig. 75) may be used as follows:

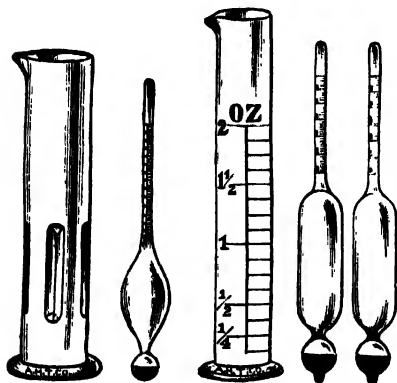


FIG. 75.—SQUIBB  
URINOMETER

FIG. 76.—VOGEL  
URINOMETER

1. Fill the cylinder without producing bubbles. Take the specific gravity without first mixing the urine.

2. Float the urinometer so that it does not *touch the bottom or sides*.

3. Make the reading from the bottom of the meniscus.

4. The instrument is usually adjusted for readings at 22.5° C. For increased accuracy, add 0.001 to the reading for each 3° C. above this temperature and subtract 0.001 for each 3° below, although moderate reduction in temperature does not influence the specific gravity as much as increased temperature.

5. The Vogel urinometer (Fig. 76) is more accurate and consists of two spindles graduated respectively from 1.000 to 1.025 and from 1.025 to 1.060.

6. In the case of urine containing large amounts of protein, correction should

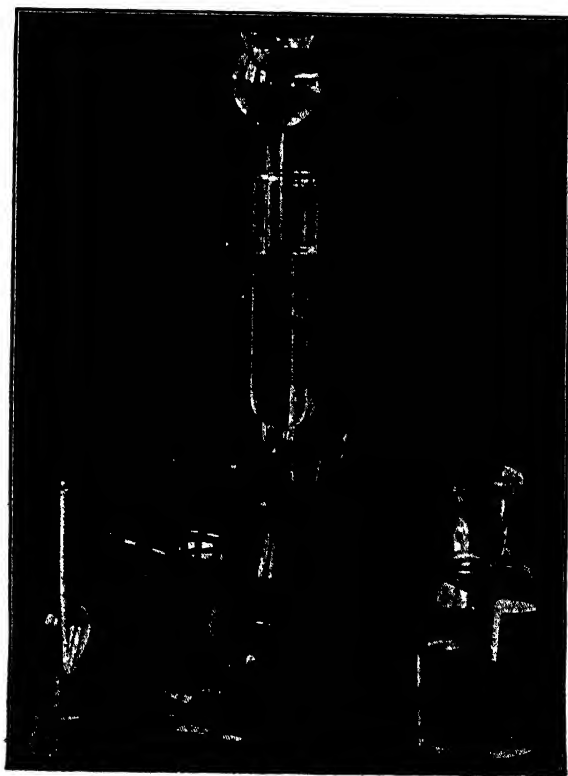


FIG. 77.—EXTON'S IMMISCIBLE BALANCE

A drop of blood is shown in suspension. A special hydrometer is shown at the left.

be made for the latter by subtracting 0.003 times grams of protein per 100 cc. of urine from the observed specific gravity.

7. For small amounts of urine, dilute with an equal volume of distilled water, mix and take specific gravity. Multiply the last two figures by 2. By this method the specific gravity is usually 0.001 to 0.002 higher. The Saxe urino-pyknometer (Eimer and Amend) may be used if at least 3 cc. of urine is available.

8. The Exton immiscible balance (Fig. 77) supplied by the Emil Greiner Company, New York, may be employed for determining the specific gravity of drops of urine when only very small amounts are available, as in ureteral catheterization. The method and instrument are based on the principle of suspending the urine in an immiscible medium of the same specific gravity, which is then determined by the usual methods, making possible rapid manipulations with minimal loss of material and accurate determinations.

The cylindrical mixing chamber is partly filled with a mixture of varuolene (petroleum ether) and carbon tetrachloride with a specific gravity of about 1.012. The side thistle tube is filled with varuoline and the carbon tetrachloride is kept at hand in a drop bottle. A drop of the urine is immersed in the mixture which is then easily and rapidly varied by means of the stopcock and dropper, so that the urine remains suspended near the middle. The specific gravity of the mixture is then taken by means of the Exton hydrometer supplied with the instrument, which gives the specific gravity of the urine.

## METHOD FOR ESTIMATION OF TOTAL SOLIDS

**Principles.**—1. Under normal conditions the total solids present in about 1500 cc. of urine varies from 60 to 70 grams for a young adult of 150 pounds. After the forty-fifty year age period they become gradually less, amounting to about half of the normal after 70 years of age.

2. The output of urinary solids is influenced by fluid intake, diet, exercise, metabolism and kidney function.

3. There is some relationship between the specific gravity and total solids of normal urine. In pathological states different substances occurring in the urine vary in the extent to which they contribute to the specific gravity; thus, less than 1.5 grams of sodium chloride will produce as great a rise in specific gravity of urine as almost 4 grams of albumin. For clinical purposes, however, it usually suffices to estimate the total solids from the specific gravity.

**Procedures.**—1. If the total output of urine for 24 hours is reported in ounces, multiply the last two figures of the specific gravity by the number of ounces voided and to the product add one-tenth of itself. This gives the total solids in grains. Example:

$$\begin{aligned}\text{Twenty-four-hour output} &= 37 \text{ ounces} \\ \text{Specific gravity (at } 25^{\circ} \text{ C.)} &= 1.014 \\ 14 \times 37 &= 518 + 51.8 = 569.8 \text{ grains}\end{aligned}$$

2. If the 24-hour specimen is reported in cc., multiply the last two figures of the specific gravity by Long's coefficient, 2.66; then multiply by the total output and divide by 1000, which gives the total solids in grams. Example:

Twenty-four-hour output = 1120 cc.

Specific gravity (at 25° C.) = 1.018

$2.66 \times 18 = 47.8$  gm. in 1000 cc. of urine

$\frac{46.8 \times 1120}{1000} = 52.4$  gm. in 1120 cc.

### QUALITATIVE TESTS FOR ALBUMIN

**Principles.**—1. Proteins, especially albumin and the globulins, are among the most important of the organic constituents occurring in the urine. The quantity of albumin usually exceeds that of the globulins and while the term “proteinuria” is more exact than “albuminuria”, yet the latter term is used commonly to designate that group of protein substances responding to the commonly employed qualitative and quantitative tests for albumin.

2. Normal urine may contain a minute amount of protein (about 0.075 gm. per 24-hour output) but this is too slight for detection by the simple tests in general use except when Exton’s reagent is used.

3. All methods depend upon the precipitation of protein by chemical agents or coagulation by heat. Most are subject to some error largely due to the precipitation of mucin or other constituents. When a considerable amount of mucin is present it can be removed by acidifying with acetic acid and filtration.

4. *Urine to be tested for albumin should be clear*, preceded by filtration or centrifugation, if necessary, in order to detect small amounts of albumin. As a general rule, simple filtration through ordinary filter paper is sufficient unless cloudiness is due to bacteria. Very large numbers of bacteria, especially dissolved organisms in alkaline urine, may yield faint traces of albumin. They are difficult to remove but this may be accomplished sufficiently for testing by centrifuging or by adding about 1 teaspoonful of purified talc, infusorial earth or animal charcoal to each 2 or 3 ounces, shaking well and filtering through two thicknesses of filter paper. Some albumin is also removed by adsorption.

5. In extremely concentrated urine certain of the salts may interfere with tests for albumin. In such cases dilution of the urine will render the test more definite, even though the concentration of albumin is thereby reduced.

6. Albuminous urine foams markedly on shaking and the foam remains a long time. This gives a rough indication of the presence of albumin before tests are made.

**Methods for Recording Reactions.**—A wide diversity of methods for reporting qualitative tests is in use which accounts, in large part, for discrepancies in reports from different laboratories. A uniform method and terminology are urgently needed. The following are recommended:

— = *negative*

± = *very slight trace*. Cloudiness or ring can just be seen against a black background (0.01 per cent or less).

+ (1) = *slight trace*. Cloud is distinct but not granular; no definite flocculation. Or, the ring is sufficiently definite to be seen without a black background (0.01 to 0.05 per cent).

- ++ (2) = *moderate trace*. Cloud is distinct and granular without definite flocculation. Or, the ring is dense but not wholly opaque when viewed from above (0.05 to 0.2 per cent).
- +++ (3) = *heavy cloud*. Cloud is dense with marked flocculation or the ring is heavy, wholly opaque and sometimes curdy (0.2 to 0.5 per cent).
- ++++ (4) = *very heavy cloud*. Heavy precipitate to boiling solid; or very dense ring. Represents 0.5 or higher per cent of albumin; 3 per cent albumin boils solid.

**Heat and Acid Tests.**—1. Boil about 5 cc. of clear urine in a thin walled test tube for 1 or 2 minutes in a Bunsen flame. An alcohol lamp may be used. The tube may be held with a test tube clamp, or simply with a strip of paper. Otherwise, boiling may be conducted in a water bath.

2. Add 3 to 5 drops of 5 per cent acetic acid, a drop at a time, or 1 to 3 drops of concentrated nitric acid.

3. A white cloud or flocculent precipitate (which usually appears during the boiling, but if the quantity of albumin be very small only after the addition of the acid) denotes the presence of albumin. The addition of too much acid may dissolve faint traces of albumin and give a falsely negative reaction.

4. A rough estimate of the amount of albumin present may be gained when the tube is allowed to stand over night. When the entire specimen coagulates, the albumin usually amounts to 2 to 3 per cent. Sediments reaching  $\frac{1}{2}$ ,  $\frac{1}{3}$ ,  $\frac{1}{4}$  and  $\frac{1}{10}$  the height of the column of urine correspond respectively to about 1.0, 0.5, 0.25 and 0.1 per cent albumin. When there is only a slight cloudiness the albumin does not usually exceed 0.01 per cent.

5. A similar white precipitate, which disappears upon the addition of the acid, is due to earthy phosphates. Effervescence upon the addition of the acid is generally due to carbonates from the food, notably lemonade.

6. Resinous drugs might give a white cloud which disappears upon the addition of alcohol.



FIG. 78.—BOILING URINE

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, The Williams and Wilkins Co., Baltimore.)

7. A white cloud which appears only after cooling may be due to Bence-Jones protein or to primary proteose.

**Purdy's Test.**—1. Fill a thin walled test tube about  $\frac{2}{3}$  full of urine.

2. Add about  $\frac{1}{6}$  its volume of a saturated solution of sodium chloride (to raise the specific gravity and prevent the precipitation of mucin) and 5 to 10 drops of 50 per cent acetic acid.

3. Mix well and boil the upper portion over a Bunsen burner (Fig. 78). A holder is unnecessary. Rotate or shake gently while heating to prevent cracking of the tube by condensation of steam.

4. A white cloud in the heated portion shows the presence of albumin. A faint cloud is best seen when viewed against a black background (Fig. 79). Bence-Jones protein may produce a white cloud, which disappears upon boiling and reappears upon cooling.



FIG. 79.—CLOUD OF ALBUMIN SEEN AGAINST A DARK BACKGROUND

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, The Williams and Wilkins Co., Baltimore.)

**Sulphosalicylic Acid Test.**—1.

Place 1 cc. of urine in a test tube. If urine is not clear it should be filtered.

2. Add 1 cc. of the reagent.

#### REAGENT

Sulphosalicylic acid . . . . . 30 gm.  
Water to make . . . . . 1000 cc.

3. Allow to stand 10 minutes.

4. If cloudiness does not develop, albumin is absent and the reaction is negative. Any cloudiness indicates the presence of albumin, the density depending upon the amount present. The reaction may then be recorded by the symbols described above.

If the urine is cloudy and cannot be cleared, the reaction should be compared with a tube containing water and urine. A distinct difference will be noted if albumin is present. This test

is quite sensitive and highly recommended for routine work.

**Exton's Test.**—1. Prepare the reagent by dissolving 50 gms. of anhydrous sodium sulfate in 800 cc. of water with the aid of heat. Cool to 35° C. and add 50 gms. of sulfosalicylic acid. Dissolve and dilute to 1000 cc.

2. Mix equal volumes of clear urine and reagent in a test tube. Warm the mixture gently; do not boil.

3. If cloudiness does not develop in the cold, albumin is absent. If cloudiness appears and persists or increases on gentle heating, albumin is present.

4. The reaction is read while the mixture is warm, since secondary proteoses will cause a clouding when it cools. The Bence-Jones protein causes a heavy precipitate which clears partially or wholly upon boiling.

**Osgood-Haskin's Test.**—1. To 5 volumes of urine in a test tube, add 1 volume of a 50 per cent solution of acetic acid, followed by 3 volumes of a saturated aqueous solution of sodium chloride.

2. Heat the mixture gradually to boiling.

3. A precipitate appearing upon the addition of the acid indicates bile salts, urates, or resin acids, etc., whereas a precipitate appearing after the addition of the salt solution suggests *Bence-Jones protein*, or globulin in excess of 0.38 gm. per liter. As the temperature is raised, the precipitate of Bence-Jones protein, if present, will go back into solution; if albumin or globulin are present, a precipitate will form. Therefore, this test has the advantage of indicating the presence of Bence-Jones protein as well as albumin and globulin.

**Robert's Test.**—1. Prepare the reagent by adding 1 volume of concentrated nitric acid to 5 volumes of a saturated aqueous solution of magnesium sulfate (U.S.P.).

2. Place a few cubic centimeters of reagent in a test tube, tilt the tube, and introduce the urine with a pipet, allowing the urine to flow gently down the side of the tube so as to overlay the reagent without mixing. If albumin is present, a fluffy, white ring of precipitated albumin forms at the line of contact.

3. Or, the test may be conducted with a pipet made of glass tubing with an inside diameter of about 5 mm. Place a few cubic centimeters of the reagent in a test tube. With the pipet take up a small column of urine, about 1 cm. long, wipe excess of urine from the outside, then place the pipet in the test tube carefully holding the finger firmly over the upper end until the other end touches the bottom of the tube. Release the finger pressure gradually, allowing the reagent to rise in the pipet, forming a clear, distinct layer with the urine.

4. A white ring at the junction of the reagent and urine, by either method, indicates albumin, the thickness and density of the ring showing the amount. No confusing color rings due to indican, iodides, bile pigments or the oxidation products of organic constituents are formed, as is frequently the case when nitric acid alone (Heller's test) is used. A white ring or cloudiness may form above the contact zone, due to urates or mucus, but such rings are less sharp, broader, and lie above the albumin ring when both are present.

5. It is advantageous to adopt a standard time of 1 minute for observation of the ring. According to its color, the ring is seen most clearly if viewed against a white or a black background, as the case may be; or one side of the test glass may be painted half white, half black, for this purpose. It is better, however, to use clear glass and to have the white or black background at a distance of several feet.

## QUANTITATIVE TESTS FOR ALBUMIN

**Exton's Test.**—1. Prepare the reagent by dissolving 50 gms. of sulfosalicylic acid and 50 gms. of anhydrous sodium sulfate in about 800 cc. of distilled water. Add 25 cc. of 0.4 per cent aqueous solution of bromphenol blue. Make up to 1000 cc. with distilled water and filter through acid-washed paper.

2. Place 3 cc. of urine in a test tube and add 3 cc. of reagent. The tube should be scrupulously clean and of the same dimensions as the standard tubes.

3. Allow to stand for 5 minutes. Warm the mixture by passing the tube slowly through a flame several times, but do not boil.



4. The reaction is turbidimetric and the quantity of albumin is read directly by comparison with a set of standard tubes representing 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg. protein per 100 cc. of urine. These standard tubes may be obtained from the Standard Reagents Company, Philadelphia, Pa. Make all suspensions homogeneous in the standard tubes by inverting gently. If necessary, make the reading between any two of the standard tubes.

5. If the urine contains more than 100 mg. protein per 100 cc., dilute the mixture of urine and reagent with a diluting solution prepared by dissolving 10 gms. sodium sulfate in 800 cc. of distilled water and adding 5 cc. of concentrated sulfuric acid, 25 cc. of 0.4 per cent aqueous solution of bromphenol and distilled water to give a total volume of 1000 cc. Make another reading and multiply the reading by the dilution.

6. To make the test on cloudy urines which cannot be cleared, dilute the urine with an equal volume of diluting solution and read against the standard tubes. Precipitate a similar volume of urine in the usual way and read. The difference between the reading due to turbidity and that after precipitation represents the amount of albumin.

7. Urines containing large amounts of carbonates or other alkaline salts must be made acid before testing in order to prevent frothing.

8. Because bromphenol blue is also an indicator on the alkaline side, to prevent decomposed or alkaline urines from turning purple, they must be made acid before testing.

9. If the color is very dark or unusual, the dilutions must be made with the diluting solution.

10. If a precipitate resembling curdled milk appears when the reagent is added, the urine contains Bence-Jones protein. This may be proved by the *disappearance* of the precipitate on boiling and its *reappearance* as the solution cools again.

**Life Insurance Test.**—A somewhat similar method devised by Kingsbury and his colleagues<sup>1</sup> has been adopted by the Committee on Urinary Impairments of the Association of Life Insurance Directors of America.

1. Permanent standards may be secured from the Standard Reagents Company, Philadelphia, Pa., or prepared as follows: Dissolve 20 gm. of purest sheet gelatin in 120 to 140 cc. of distilled water at 45° to 55° C. and make up to 200 cc. Add half of the white of an egg and stir it in thoroughly. Heat in a boiling water bath for at least 30 minutes after a temperature of 90° C. has been attained. Filter hot through a Whatman No. 4 paper, yielding a perfectly clear, slightly yellow solution. Immediately before use, add 0.3 cc. of formalin to each 100 cc. of gelatin solution. Formazin, the substance to be suspended in the gelatin, is made up as follows: Dissolve 2.5 gm. of urotropin (hexamethylene tetramine) in 25 cc. of distilled water at room temperature. Add this to 25 cc. of 1 per cent hydrazine sulfate solution also at room temperature. Mix, stopper, and allow to stand at least 15 hours. Suspend the white amorphous precipitate uniformly by gently inverting the flask several times. Add 14.5 cc. of the formazin suspension to 100 cc. of the 10 per cent gelatin solution, to which the correct amount of formalin has been added, at 45° to 55° C. and mix thoroughly. This produces a turbidity equivalent to that made by an albumin solution of 0.1 per cent, or 100 mg. in 100 cc., when precipitated by 3 volumes of 3 per cent sulfosalicylic

acid. Dilute the stock suspension according to the following table to make the other standards required:

Stock Formazin Suspension Equivalent to 100 mg. Albumin per 100 cc.	10 per cent Clarified Gelatin	Value of Standard Made	
		Per cent	Mg. per 100 cc.
37.5 cc.	12.5 cc.	0.075	75
25.0 "	25 "	0.05	50
20.0 "	30 "	.04	40
15.0 "	35 "	.03	30
10.0 "	40 "	.02	20
5.0 "	45 "	.01	10
2.5 "	55 "	.005	5

Pour each standard into a test tube of the same dimensions as those used in making the test with urine. Seal the tubes with waxed stoppers and allow to cool to room temperature. Keep in a well lighted room. In extremely hot weather, keep in a cool place. If in time they become greenish, exposure to sunlight will bleach them. There is no appreciable change in turbidimetric value in 6 to 8 months and only a slight change in a year. It is best to replace them after 10 months.

2. Pipet 2.5 cc. of urine, cleared by filtration or centrifugalization, into a test tube graduated at 10 cc. and add a 3 per cent solution of sulfosalicylic acid in distilled water to the 10 cc. mark. Invert several times, allow to stand for 10 minutes, and compare the turbidity with that of the permanent standards. Record the value of the standard most closely matched as the albumin content of the urine.

For values above 100 mg. per 100 cc., repeat the test with a portion of urine sufficiently diluted with water to come within the range of the standards; multiply the result obtained by the dilution.

**Shevsky-Stafford Test.**—1. Prepare the Tsuchiya reagent by mixing 15 gms. of phosphotungstic acid, 50 cc. of concentrated hydrochloric acid, and 1000 cc. of 95 per cent ethyl alcohol.

2. If the urine contains considerable albumin, dilute 1 cc. with 9 cc. of water. In urines with very scant albumin it is not necessary to dilute. Occasionally a urine is encountered with more than 2.8 per cent albumin, which is the maximum that can be determined with a 1:10 dilution. In such a case dilute 1 cc. of urine with 19 cc. of water.

3. Place 4 cc. of urine (diluted or undiluted) into a special graduated centrifuge tube (A. H. Thomas Co., No. 3007A). Add the reagent to the 6.5 cc. mark. Mix the contents thoroughly by inverting the tube several times, allow to stand exactly 10 minutes, and centrifugalize for exactly 10 minutes at 1800 r.p.m. The volume of precipitate is read on the scale in hundredths of a cubic centimeter.

4. Calculate as follows: Grams of albumin per 1000 cc. of urine = cc. of precipitates  $\times 7.2 \times$  dilution, where dilution indicates the number of times the urine was diluted before the sample was measured into the tube.

**Esbach's Test.**—1. Prepare the reagent by dissolving 10 gms. of citric acid in 1000 cc. of water. Or, the reagent may be prepared by diluting 100 cc. of trichloro-

cetic acid with 900 cc. of water. The latter is preferred because the effects of the temperature and specific gravity of the urines are reduced to a minimum.

2. Fill an Esbach-Quick albuminometer (Fig. 80) with urine to the mark U.

Add reagent to the mark R. Close with a rubber stopper, invert several times and set aside in a cool place for 18 to 24 hours.

3. Read off the results according to the markings on the tube which show albumin in grams per 1000 cc.; to express the per cent, divide by 10.



FIG. 80.—ESBACH-QUICK-ALBUMINOMETER

### TEST FOR BENCE-JONES PROTEIN

**Principles.**—1. Bence-Jones protein is characterized by the formation of a precipitate when urine is heated at 50° to 60° C. It wholly or partially disappears as the temperature approaches the boiling point and reappears upon cooling. It is markedly influenced by acidity and salt concentration.

2. Bence-Jones protein occurs in the urine in a large percentage of cases of multiple myeloma as likewise in some cases of osteogenic sarcoma, osteomalacia, carcinomatous metastases of the bone marrow and leukemia.

3. As previously stated, it may be detected in the heat and acid, Purdy, Exton and Osgood-Haskins qualitative and the Exton quantitative tests for albumin. A special test may be conducted as follows:

**Procedure.**—1. Place the urine in a water bath with a thermometer and heat very slowly and gently.

2. Observe frequently. Turbidity will begin to occur at about 40° C. and precipitation will take place at about 60° C.

3. Now acidulate *very slightly* with acetic acid and raise the temperature to the boiling point (100° C.). The precipitate now partly or totally disappears.

4. Allow to cool and if Bence-Jones protein is present the precipitate will reappear.

5. Urine containing albumin should be filtered at or near the boiling point, the aforementioned procedures being applied to the filtrate.

6. If the test is positive it is advisable to confirm the results by one or both of the following tests:

(a) Precipitate the protein with nitric acid. This precipitate should disappear on boiling and reappear upon cooling.

(b) Precipitate the protein with alcohol and collect immediately by centrifuging. The precipitate should be soluble in water.

### TEST FOR PROTEOSES

**Principles.**—Proteoses, particularly deuteroproteose and heteroproteose, have frequently been found in the urine in the presence of considerable autolysis and absorption of exudates and tissues. They are divided into two groups, namely, *primary and secondary*. The *primary proteoses* are precipitated upon half saturation with ammonium sulphate and the *secondary proteoses* upon complete saturation.

**Procedure.**—1. Acidify the urine with acetic acid and filter off any precipitate of nucleoprotein which may form.

2. Boil for several minutes.
3. Filter while hot to remove the albumin and globulin.
4. Test the cooled filtrate by overlaying a saturated solution of trichloroacetic acid. A white ring at the point of contact indicates the presence of proteoses.
5. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulfate.

### TEST FOR MUCIN

**Principles.**—The exact chemical nature of mucin has not been determined but it appears to be a glycoprotein. Traces may be present in normal urine. It is frequently increased in irritations and inflammations of the mucous membrane of the urinary tract or vagina. Upon boiling with an acid or alkali, as in Fehling's test, it may yield a carbohydrate which reduces copper. Mucin must be differentiated from the Bence-Jones protein.

**Procedure.**—1. Dilute the urine with about 3 volumes of water and make strongly acid with glacial acetic acid. Do not heat.

2. The presence of turbidity indicates a positive reaction. Albumin is not precipitated by this concentration of acetic acid.

### TEST FOR TOTAL NITROGEN

**Principles.**—1. The *total nitrogen* of urine comprises mostly the nitrogen of urea, uric acid, creatine, creatinine and amino acids. On a mixed diet it averages 12 to 18 gms. per 24-hour urine. The total nitrogen varies directly with the protein ingested and usually parallels the excretion of urea nitrogen.

2. In the urine *uric acid* exists as the urates of sodium, potassium or ammonium, or in the free state, averaging about 0.4 to 1.0 gm. per 24-hour urine under average normal conditions.

3. *Creatine* is not usually present in the urine of normal adults but may occur in males in varying amounts up to 0.196 gm. per 24-hour urine estimated as creatine nitrogen.

4. *Creatinine* occurs normally in the urine in an amount approximately about 1.25 gm. per 24-hour urine. It is quite constant and independent of diet, provided no preformed creatinine is ingested. Its excretion is usually in relation to muscular activity.

5. *Amino acids* are eliminated in the urine in both the combined and free state, amounting to a total of 0.5 to 1.0 gm. per 24-hour urine, of which 0.1 to 0.15 gm. is free amino acid nitrogen. Increased urinary excretion is especially found in severe hepatitis with necrosis as well as in wasting states.

6. Upon a mixed diet, under normal conditions, the *partition of nitrogen* is somewhat as follows: 86.9 per cent urea nitrogen; 4.4 per cent ammonia nitrogen; 0.75 per cent uric acid nitrogen; 3.6 per cent creatinine nitrogen and 4.3 per cent "undetermined nitrogen", chiefly amino acids. A determination of nitrogen partition is not usually of clinical value.

**Haden's Modification of the Folin-Dennis Method.**—1. Measure the volume of the 24-hour specimen; take the specific gravity and test for albumin.

2. Pipet 5 cc. urine into a 100 cc. volumetric flask. (The pipet is to be drained 15 seconds against the wall of the flask and then blown.)
3. Dilute to the mark with distilled water and mix.
4. With a pipet, transfer 1 cc. of the diluted urine to a dry, 200 by 25 millimeters, thin-walled, lipped Pyrex test tube graduated at 35 cc. and 50 cc.
5. Add a dry glass bead and 1 cc. digestion mixture.
6. Proceed as under Determination of Non-Protein Nitrogen of the Blood (see page 52).
7. Set the urine sample at 15 mm. in the colorimeter, when the reading of the standard multiplied by 0.02 = grams of total nitrogen per 100 cc.

$$\text{Grams total nitrogen per 100 cc.} \times \frac{\text{cc. volume of 24-hour specimen}}{100}$$

= Grams total urinary nitrogen per 24 hours

### TESTS FOR UREA, UREA NITROGEN AND AMMONIA NITROGEN

**Principles.**—1. The estimation of urea or urea nitrogen is of but little clinical value when a small quantity of urine taken at random is used. Estimations under the same conditions of diet and exercise, however, are of much value *providing a sample of the mixed 24 hour urine is used.*

2. Neither albumin nor sugar, nor any other substance likely to be present, interferes with the action of urease in determinations for urea or urea nitrogen.

3. Normally, *urea* varies from 20 to 30 gms. and *urea nitrogen* from 12 to 16 gms. per 24-hour urine on an average diet.

4. The urine must be fresh in estimations of *ammonia* since decomposition of urea increases the free ammonia after the urine has been voided. In terms of  $\text{NH}_3$  about 0.6 gm. is normally present per 1000 cc. of urine.

**Marshall's Urease Test for Urea.**—1. Into each of two 200-cc. flasks measure 5 cc. of urine and about 100 cc. of water. Add two 0.025 gm. tablets of urease (Hynson, Westcott and Dunning, Baltimore) crushed and dissolved in 5 cc. of water.

2. Overlay the fluid in each flask with about 1 cc. of toluol, insert corks, and let stand overnight at room temperature (or for 3 hours in the incubator at 37° C.).

3. At the end of that time titrate the contents of each flask to a distinct pink color with decinormal hydrochloric acid, using a few drops of 0.5 per cent methyl orange solution as indicator.

4. Find the difference between the number of cubic centimeters of decinormal acid used in the two titrations and multiply this by the factor 0.06 to obtain the amount of urea per 100 cc. of urine. From this calculate the total amount in the 24-hour output of urine.

**Van Slyke's and Cullen's Test for Urea, Urea Nitrogen and Ammonia.**—

1. Prepare *urease paper* as follows: Transfer 30 gms. of jackbean meal to a 200 cc. flask, add 100 cc. of dilute ethyl alcohol (30 cc. of 95 per cent ethyl alcohol diluted to 100 cc.) and 1 cc. of acetate buffer. Stopper and shake vigorously for 15 minutes. Transfer to centrifuge tubes, close the mouths with tin foil, and centrifugalize for 30 minutes. Transfer the supernatant to a flat-bottomed dish and take up at once on strips of filter paper (Schleicher and Schull's No. 597). Suspend the papers and allow

to dry over night in an incubator at  $37.5^{\circ}\text{C}$ . Cut into pieces about  $1 \times 2.5\text{ cm.}$  and preserve in wide-mouthed dark glass bottles. Urease so prepared will retain its activity for at least 6 months.

2. Prepare *acetate buffer* by dissolving 15 gms. of crystallized sodium acetate in a 100 cc. volumetric flask with 50 to 75 cc. of water. Add 1 cc. of glacial acetic acid, dilute to volume, and mix.

3. Prepare five aeration tubes, 200 by 25 mm. with inlet and outlet tubes, two-holed stoppers, and rubber tubing connections for aeration as shown in Figure 81. Mark the tubes A, B, C, D and E in the order in which the stream of air will pass through.

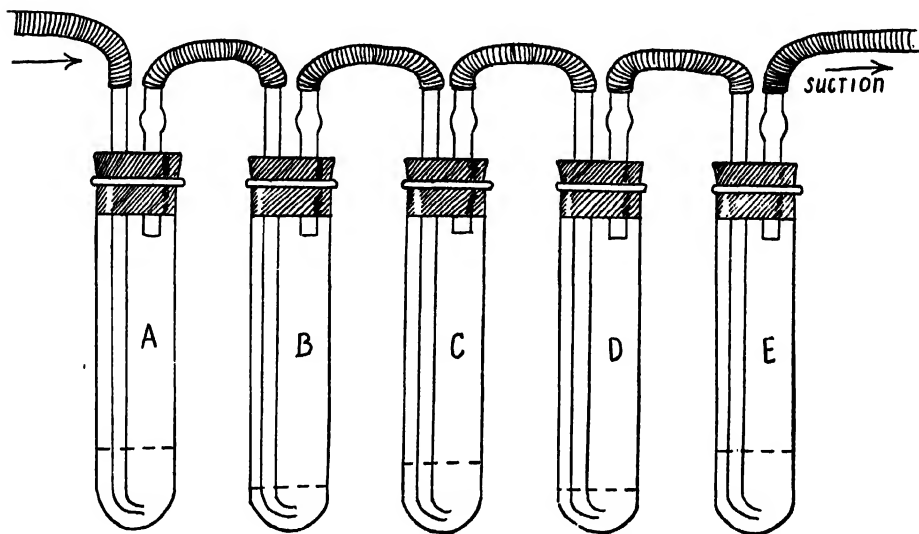


FIG. 81.—AERATION APPARATUS

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

4. In tube A place 20 cc. of dilute sulfuric acid; in tube B 10 cc. of ammonia-free water, 2 drops of acetate buffer and a piece of urease paper; in tubes C and D 25 cc. of 0.02 N sulfuric acid and 5 drops of methyl red indicator; in tube E 5 cc. of urine and 5 drops of caprylic alcohol.

5. Insert stoppers tightly making sure that the inlet tubes reach nearly to the bottom of each tube. Add 5 drops of caprylic alcohol and exactly 0.5 cc. of urine to tube B. Stopper quickly. Allow to stand 20 minutes or longer, occasionally shaking the tube to free the urease from the paper.

6. Attach the train of tubes to a suction pump and draw air through all the tubes for 1 minute.

7. Shut off the suction and add to B and D 10 cc. of potassium carbonate solution (90 gms. dissolved in 100 cc. of distilled water). Stopper quickly and begin suction slowly, gradually increasing to a moderate rate. Aerate for at least 30 minutes; 1 hour may be required if too small a stream of air is used.

8. When the aeration is complete, turn off the suction before disconnecting the tubes (center ones first).

9. Titrate the acid remaining unneutralized in tubes C and E with 0.02 N sodium hydroxide (prepared by diluting 20 cc. of tenth normal solution with 80 cc. of distilled water).

10. The number of cubic centimeters of 0.02 N acid neutralized in tube C, multiplied by 0.056, gives the percentage of ammonia nitrogen plus urea nitrogen. The acid neutralized in tube E, multiplied by 0.0056, gives the per cent of ammonia nitrogen. The difference between the two gives the percentage of *urea nitrogen*; this figure multiplied by 2.14 gives the percentage of *urea*. To obtain the per cent of ammonia, multiply the percentage for tube E by 1.1216.

**Ormsby Test for Urea Nitrogen.**—This test (*Jour. Biol. Chem.* 146: 595, 1942) is based upon the principle that when urea is heated with biacetyl monoxide in acid solution, a yellow color develops. This color, deepened by oxidation with persulfate is compared in a photoelectric colorimeter with a standard urea solution.

1. Prepare all reagents and the standard urea solution exactly as for the blood urea nitrogen determination.

2. Dilute urine 1 to 100 with water. Place 1.0 cc. of this diluted urine in a test tube, add 2 cc. of water and proceed exactly as for the blood urea nitrogen determination (see Section on Methods for Chemical Examinations of the Blood).

3. Calculation: 
$$\frac{0.9}{\text{Reading of standard}} (= \text{factor}) \times \text{reading of unknown}$$
  
= Gm. of urea nitrogen per 100 cc. of urine.

4. Since the above reagent is not affected by ammonia, the urine need not be freed of ammonia by pre-treatment with permuted.

## QUALITATIVE TESTS FOR GLUCOSE

**Principles.**—1. The term “melituria” is employed to designate the presence of an abnormal amount of any sugar in the urine. Glucose (dextrose) is by far the most common and is termed *glycosuria*. Other sugars occasionally found in the urine are levulose (*levulosuria*), lactose (*lactosuria*), galactose (*galactosuria*), pentose (*pentosuria*), etc. Since all meliturias are not glycosurias, the identification of the sugar present in urine frequently becomes a matter of clinical importance.

2. Traces of glucose and other sugars too small to be detected by ordinary tests are present in the urine in health (*glycuresis*).

3. All sugars (glucose, levulose, lactose, galactose, pentose) readily reduce copper sulfate in alkaline solution to insoluble yellow or red cuprous oxide. Special tests are required for differentiating the sugars occurring in urine, which may be summarized as follows:

<i>Test</i>	<i>Glucose</i>	<i>Lactose</i>	<i>Levulose</i>	<i>Pentose</i>
Benedict's	+	+	+	+
Fermentation	+	—	+	—
Phenylhydrazine	Glucosazone	Lactosazone	Glucosazone	Pentosazone
Rubner's	—	+	—	—
Seliwanoff's	—	—	+	—
Bial's	—	—	—	+

### PLATE III

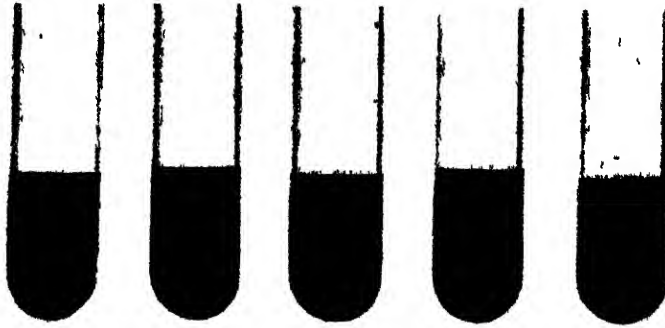


Fig. 1  
Negative

Fig. 2  
Trace of  
Sugar (+)

Fig. 3  
1 percent  
Sugar (++)

Fig. 4  
More Than  
1 Percent  
(+++)

Fig. 5  
More Than  
2 Percent  
(+++)

#### BENEDICT'S QUALITATIVE TEST FOR SUGAR IN THE URINE

(From *Essentials in the Management of Diabetes Mellitus*, Eli Lilly and Company, Indianapolis)





4. The reduction of copper in alkaline solution may be produced not only by the sugars but other substances if present in large amounts, including uric acid, nucleoproteins and conjugate glycuronates formed after the ingestion of antipyrine, menthol, phenol, camphor, chloral, etc., as well as by chloroform used as a preservative. The Benedict test is recommended not only because of its sensitiveness, accuracy and simplicity but also because the reagent is less susceptible to reduction by uric acid and chloroform.

5. If albumin is present in large amounts it may interfere with the precipitation of copper and should be removed by acidifying with acetic acid, boiling and filtering. Small amounts need not be removed.

**Benedict's Test.**—1. Prepare Benedict's qualitative reagent by dissolving 17.3 gms. of cupric sulfate crystals (U.S.P.) in about 100 cc. of distilled water. Dissolve 100 gms. of sodium carbonate (anhydrous U.S.P.) and 173 gms. of sodium citrate (U.S.P.) in 700 cc. of water, with the aid of heat, if necessary. Cool to room temperature and pour in the copper solution slowly, stirring constantly. When completely mixed, make up the volume to 1000 cc. with distilled water.

2. Place 5 cc. of reagent in a test tube. Add 0.5 cc. (not more) of urine. Boil vigorously for 3 minutes; then allow to cool. Do not hasten cooling by immersing in cold water. If a large number of tests are to be conducted, the tubes may be placed in a boiling water bath or a beaker of boiling water.

3. In the presence of glucose the entire solution will be filled with a bulky precipitate which may be greenish yellow, yellow, or red in color, depending on the amount of glucose present. In the presence of over 0.25 per cent of glucose the precipitate will form quickly. If no glucose is present the solution will remain perfectly clear or will show a faint turbidity due to precipitated urates.

4. The following scheme may be used for reporting the reactions (Plate III):

— = *negative*. No reduction. A green color with *no precipitate* is negative.

+ = *slight trace*. No reduction on boiling, but a slight greenish precipitate appears upon cooling.

+ + = *moderate trace*. Reduction after boiling about 1 minute.

+ + + = *heavy trace*. Reduction after boiling about 10 to 15 seconds.

+ + + + = *strongly positive*. Reduction occurs almost immediately upon the addition of the urine to the boiling reagent.

**Fermentation Test.**—This test depends upon the fermentation of yeast by glucose or levulose with the production of carbon dioxide. Its chief disadvantage is the time required. Its chief value is as an aid in distinguishing glucose from other sugars. The urine must be fresh with no preservative. The test is not applicable to urine undergoing ammoniacal decomposition.

1. Place 15 cc. of urine in a test tube and add a piece of fresh Fleischmann yeast about the size of a pea; mix gently to emulsify the yeast.

2. Transfer to a fermentation tube; make sure the arm is free of bubbles of air.

3. Place in an incubator for a few hours.

4. A normal urine and a normal urine to which is added a pinch of glucose may be treated in the same manner as negative and positive controls, respectively.

5. A positive reaction due to fermentation is indicated by the collection of carbon dioxide gas in the arm.

6. If necessary, guard against gas production by bacterial fermentation by adding a pinch of tartaric acid (advisable if mixtures are incubated more than 4 hours).

7. By using the Einhorn saccharometer (Fig. 82), a *quantitative* test may be conducted, as the graduations on the arm indicate with fair accuracy the percentage of glucose present from 0.1 to 1 per cent.

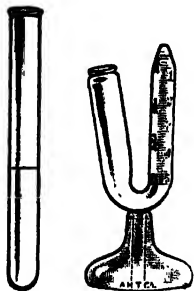


FIG. 82.—EINHORN SACCHAROMETER

**Phenylhydrazine Test (Kowarsky's Method Modified by Bluemel).**—This test is based upon the formation of yellow crystalline bodies, called *osazones*, by glucose and other sugars when brought into contact with phenylhydrazine and acetates. Each sugar forms an osazone with a definite crystalline form although glucose and levulose form the same kind of crystals. The test will detect as little as 0.025 per cent of glucose in the urine, the crystals forming in 3 to 4 hours. It is of special value in differentiating glucose from other sugars occurring in the urine.

1. In a large wide-mouthed test tube place 5 drops of pure phenylhydrazine, 10 drops of glacial acetic acid; and 1 cc. of a saturated aqueous solution of sodium chloride.

2. To the curdy mass add 3 or 4 cc. of urine and 4 or 5 cc. of water. Introduce one or more short pieces of glass tubing to prevent bumping and boil vigorously until the volume is reduced to 2 or 3 cc.

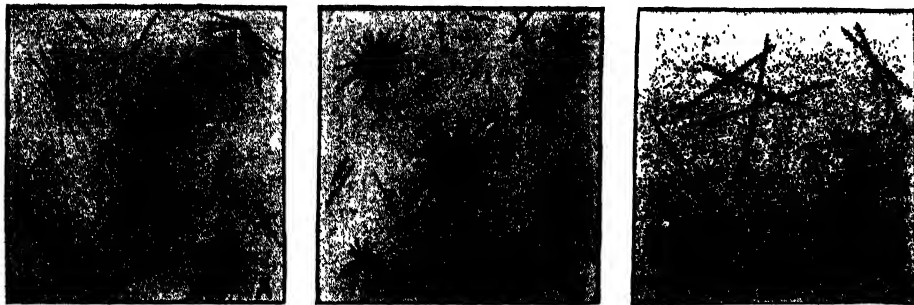


FIG. 83.—GLUCOSAZONE CRYSTALS

A, phenylglucosazone crystals; B, phenyllactosazone crystals; C, phenylpentosazone crystals.

3. Set aside to cool very slowly for 15 to 30 minutes, or longer, without agitation.

4. Examine the sediment microscopically. If glucose is present yellow, needle-like crystals arranged in clusters or sheaves are observed (Fig. 83). If only traces of glucose are present, the crystals may not appear for  $\frac{1}{2}$  hour or more.

5. The test tubes may be cleansed by boiling in a 10 per cent solution of sodium hydroxide or acetic acid.

### QUANTITATIVE TESTS FOR GLUCOSE

**Benedict's Test.**—The reagent used in this test differs from the qualitative reagent in that it contains potassium thiocyanate and potassium ferrocyanide. When reduction takes place, a white precipitate of cuprous thiocyanate is formed. The ferro-

cyanide aids in keeping cuprous oxide in solution. The reagent is stable for an indefinite length of time.

1. Prepare Benedict's *quantitative reagent* by dissolving with the aid of heat 100 gms. of anhydrous sodium carbonate in 800 cc. of distilled water. Add and dissolve 200 gms. of sodium or potassium citrate (C.P.) and 125 gms. of potassium thiocyanate (C.P.). Filter if necessary. Dissolve exactly 18 gms. of copper sulfate (C.P. crystals) in 100 cc. of distilled water and pour this solution slowly into the first one, stirring constantly. Add 5 cc. of a 5 per cent solution of potassium ferrocyanide in distilled water, cool to room temperature, and make up to 1000 cc. in a volumetric flask with distilled water. The copper sulfate should be weighed with extreme accuracy. Exactly 25 cc. of this reagent are reduced by 0.050 gm. of glucose.

2. Dilute 10 cc. of clear urine with 90 cc. of distilled water unless the glucose content is known to be low.

3. Fill a 50 cc. buret with urine (diluted or undiluted).

4. Measure exactly 25 cc. of the reagent into a porcelain evaporating dish, add about 15 gms. of crystalline sodium carbonate and 2 or 3 gms. of pumice or talc.

5. Heat to boiling over a free flame and keep the mixture boiling vigorously during the entire titration (Fig. 84).

6. As soon as the sodium carbonate is completely dissolved, add the urine from the buret, rapidly at first, until a chalk-white precipitate forms, and the blue color begins to fade perceptibly. It is then run in a few drops at a time until the last trace of blue disappears from the solution. Half-minute intervals must be allowed to elapse between additions of urine in the final steps of the titration. Water may be added if the mixture becomes too concentrated. The end point must be determined while the solution is still hot; upon cooling, the solution tends to regain a bluish-green tint. With urine, the color of the end point tends to be a slight yellowish, or yellowish-green, due to urinary pigments.

7. When the urine is diluted 1:10 as described and N is taken as the number of cubic centimeters of diluted urine required, the calculation is made as follows:

$$\frac{0.050}{N} \times 1000 = \text{per cent of glucose.}$$

**Example:** 2480 cc. of urine voided in 24 hours

8.2 cc. urine 1:10 required to reduce 25 cc. of reagent

$$\frac{8.2}{0.050} \times 1000 = 6.1 \text{ per cent glucose or } 151.2 \text{ gms. in the 24-hour specimen.}$$

8. A short method to obtain the percentage is to divide 50 by the number of cc. of 1:10 dilution of urine required, as per the same example as follows:

$$\frac{50}{8.2} = 6.1 \text{ per cent glucose.}$$

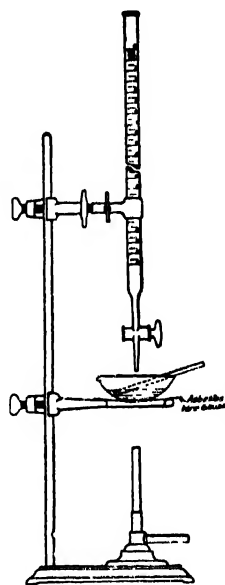


FIG. 84.—QUANTITATIVE ESTIMATION OF GLUCOSE IN URINE

9. If *undiluted* urine is used and X is taken as the number of cubic centimeters required, the calculation is as follows:

$$\frac{0.050}{X} \times 100 = \text{per cent glucose.}$$

Example: 5.2 cc. undiluted urine required

$$\frac{0.050}{5.2} \times 100 = 0.96 \text{ per cent glucose}$$

**Benedict's Test Tube Method.**—1. Place 5 cc. of Benedict's quantitative reagent in a clean test tube.

2. Add 1 or 2 grams of anhydrous sodium carbonate and an equal amount of pumice.

3. Heat to boiling over Bunsen burner.

4. While boiling run into tube from a 1 cc. pipet, graduated in 0.1 cc., undiluted urine until the last trace of blue has disappeared. The urine should be run in slowly and the solution kept boiling while the urine is being added.

5. As 5 cc. of reagent are reduced by 0.010 gram of glucose, the amount of urine used contains this amount.

6. To obtain the percentage, divide 100 by the amount of urine and multiply by 0.010:

$$\begin{array}{l} 0.8 \text{ cc. urine required} \\ \frac{100}{0.8} \times \frac{0.010}{1} = 1.25 \text{ per cent} \end{array}$$

7. Or divide 1 by the amount of urine to obtain the percentage:

$$\frac{1.0}{0.8} = 1.25 \text{ per cent}$$

8. If the urine contains a large amount of sugar, dilute 1:10 and calculate accordingly.

**Sumner's Method.**—1. Into a small test tube, pipet 1 cc. of the urine to be examined; add 9 cc. of water with a pipet or by diluting to a 10 cc. mark on the tube. Mix.

2. Pipet 1 cc. of this diluted urine into a Folin sugar tube or to a test tube graduated at 25 cc.

3. Add 3 cc. of the reagent and place in boiling water for 5 minutes. Cool in running water, dilute to mark, and mix.

4. Compare with the standard tubes and read directly the percentage from the tube. In case it is stronger than 3 per cent, dilute with a definite proportion of water, generally equal parts, and multiply the result by the dilution factor.

**Reagent.**—Dissolve 10 grams of dinitrosalicylic acid in water and dilute to 1 liter.

Place 300 cc. of 4.5 per cent sodium hydroxide in a 2 liter graduated cylinder; add 880 cc. of 1 per cent dinitrosalicylic acid and 255 grams of sodium potassium tartrate. Mix until dissolved. Preserve in a brown bottle in the dark.

**Permanent Standard Series.\***—Dissolve 0.8625 gram of ferric ammonium sulphate in water and dilute to 100 cc. in a volumetric flask. Into each of twelve 100 cc. volu-

\* These standards, in hermetically sealed tubes, may be obtained from the Standard Reagents Company, Philadelphia, Pa.

metric flasks add 10 cc. of the 1 per cent dinitrosalicylic acid solution and add in order 1, 1.75, 2.53, 3.22, 4, 4.68, 5.32, 6.10, 6.80, 7.5, 9.1, 10.3 cc. of ferric ammonium sulphate solution. Dilute each to 100 cc. and mix. Transfer portions of each to tubes similar to those used in doing the test and label these tubes respectively, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.5, 3 per cent. These tubes will then read directly in percentage of sugar considering the 1:10 dilution as made in the test.

*Notes.*—1. Slightly increased accuracy may be obtained by using an artificial or glucose standard and the colorimeter.

2. In the above method the qualitative test may be combined with the quantitative. All urines reading 0.2 per cent or under may be considered *negative* for glucose.

3. Sugar in normal urine may be read by using undiluted urine in the above test when the percentage of sugar will be that given on the standard tubes divided by 10.

4. Urines showing 0.25 per cent or over will give a positive Benedict qualitative test.

### RUBNER'S TEST FOR LACTOSE

**Principles.**—Lactose is found occasionally in the urine of women during lactation and in patients who have been on an exclusive milk diet for a long time. It reduces copper solutions, although less actively than glucose, 0.0676 gm. being required for the reduction of 25 cc. of Benedict's quantitative reagent. Lactose does not ferment yeast although bacteria may hydrolyze it into its constituents, glucose and galactose. In large amounts it can form phenyllactosazone crystals (Fig. 83) but is unlikely to do so when the test is applied directly to the urine.

**Procedure.**—1. To 10 cc. of undiluted urine add 3 gms. of lead acetate (excess).

2. Shake well and filter into a test tube.

3. Boil the filtrate for a few seconds; add 1 cc. of concentrated ammonium hydroxide and boil again.

4. If lactose is present, the solution turns brick-red and a red precipitate develops which is the criterion.

5. This test is not very sensitive but will detect lactose in about 0.3 to 0.5 per cent.

6. Glucose gives a red solution with a yellow precipitate.

A modified test is conducted as follows: 1. Place 3 cc. of urine in a test tube; add 2 cc. of concentrated ammonium hydroxide and 3 drops of a 10 per cent solution of sodium hydroxide.

2. Heat in a beaker of boiling water and observe after 2, 3, 4 and 5 minutes of heating.

3. A distinct reddish but not brilliant color is a positive reaction. Heating too long diminishes the color and a brownish tinge may appear. Other sugars give a yellow color. Lactose must be present in over 0.2 per cent for positive reactions.

### BIAL'S TEST FOR PENTOSE

**Principle.**—This test depends upon the production of a greenish flocculent precipitate of furfural when pentose is heated in a strongly acidified solution of orcinol. If glucose is present it must be first removed by fermentation with yeast. Pentosuria may be alimentary and temporary or pathological (especially in diabetes mellitus).

**Procedure.**—1. Prepare the reagent by dissolving 1.5 gms. of orcinol in 500 cc. of 30 per cent hydrochloric acid and adding 1 cc. of a 10 per cent solution of ferric chloride.

2. Test for glucose (Benedict's qualitative test) and, if present, first remove it by fermentation and filter.

3. Place 5 cc. of the reagent in a test tube and add 2 cc. of urine.

4. Mix and heat gently until the first few bubbles rise to the surface. Or, the urine may be added to the hot reagent, in which case no further heating is necessary.

5. If a pentose is present, the solution immediately, or upon cooling, becomes green and a flocculent precipitate of the same color may form.

### TESTS FOR LEVULOSE

**Principles.**—Levulose (fructose), or fruit sugar, is seldom present in urine except in association with glucose. Its name is derived from the fact that it rotates polarized light to the left. It may also appear in the urine after the ingestion of large amounts of honey. Levulose responds to all tests for glucose and produces the same osazone with phenylhydrazine as glucose (Fig. 83). It may be distinguished from glucose by the following tests, depending upon the production of a red colored precipitate when levulose is heated in a strongly acidified solution of resorcinol.

**Borchardt's Test.**—1. Place 5 cc. of urine in a test tube and add 5 cc. of 25 per cent hydrochloric acid (conc. HCL, 2 parts; water, 1 part).

2. Add a few crystals of resorcinol.

3. Boil for not more than  $\frac{1}{2}$  minute. (If levulose is present, a red color appears.)

4. Cool in running water, pour into a beaker and render slightly alkaline with solid sodium or potassium hydroxide.

5. Pour back into test tube, add 2 or 3 cc. of acetic ether, and shake.

6. If levulose is present, the ether will be colored yellow.

The administration of rhubarb or senna will cause a yellowish coloration of the ether similar to levulose. If indican is present it should be removed by doing the Obermayer's test. After the chloroform has extracted the indican, remove the supernatant fluid and reduce the acidity by adding one-third its volume of water. Proceed with the test omitting step 1.

**Seliwanoff's Test.**—1. Prepare the reagent by dissolving 0.15 gm. resorcinol in a mixture of 100 cc. of reagent hydrochloric acid and 200 cc. of distilled water.

2. Place 5 cc. of reagent in a test tube, add 6 to 8 drops of urine and heat to boiling. *Boil for not more than 20 to 30 seconds.* Note the color of the fluid and whether or not a precipitate forms. If a red precipitate is present, pour off the supernatant fluid and add 4 or 5 cc. of 95 per cent ethyl alcohol. Mix and note the color of the alcohol.

3. A positive reaction is indicated by the production of a red color and the separation of a red precipitate after not more than 20 to 30 seconds of boiling. The precipitate must dissolve in alcohol and give it a bright red color. More than 2 per cent glucose will give a positive reaction.

### TEST FOR GALACTOSE

**Principles.**—Galactose may occur in the urine of nursing infants with severe disturbance of the digestive tract. It may be differentiated from other reducing sugars (except lactose) by the formation of mucic acid.

**Procedure.**—1. Place 100 cc. of urine in a broad, shallow, evaporating dish.

2. Add 20 cc. of nitric acid and evaporate over boiling water until volume is about 20 cc. (If the specific gravity of the urine is over 1.020, add 25 to 35 cc. of acid and evaporate to a volume equal to the volume of acid used.)

3. If galactose is present, the fluid will be clear with a fine white precipitate of mucic acid. Lactose may give a positive reaction.

## TESTS FOR ACETONE

**Principles.**—1. Minute traces of acetone, too small for detection by ordinary tests, may be present in the urine under normal conditions. This is especially true of young children in whom larger amounts are apt to occur in the urine.

2. Large amounts are not uncommon in severe diabetes mellitus, when the intake of carbohydrates is limited in fevers, in gastro-intestinal disorders, the toxemias of pregnancy and after anesthesia. In surgical cases it is a good routine practice to test for acetone before operations. According to Polin, acetone is usually present in only small amounts in these conditions, the substance found, particularly after distillation of the urine, being diacetic acid.

3. The urine may be tested directly, but it is much better to distil it after adding a little phosphoric or hydrochloric acid to prevent foaming and to test the first few cubic centimeters of the distillate. When diacetic acid is present, a considerable proportion will be converted into acetone during distillation.

4. In nitroprusside tests the detection of acetone is based upon a reddish-purple reaction due, presumably, to the formation of ferropentacyanide with the isonitro compound of the ketone or the formation of such an ion with the isonitroamine derivative of the ketone.

**Ross Modification of Rothera's Test.**—1. Prepare the reagent by mixing 1 gm. of powdered sodium nitroprusside with 100 gms. of powdered ammonium sulfate.

2. Place 1 gm. of the dry powdered reagent in a test tube and add 5 cc. of filtered or distilled urine. Mix until the powder is dissolved. Then overlay with a 28 per cent solution of ammonium hydroxide.

3. A red-purple permanganate color at the line of contact indicates the presence of acetone.

**Lange's Test.**—1. Prepare a *fresh* saturated solution of sodium nitroprusside by dissolving several crystals in 1 to 2 cc. of water by gentle heat, having a slight excess of undissolved crystals remaining.

2. Place 5 cc. of filtered or distilled urine in a test tube. Add 0.5 cc. of glacial acetic acid and 0.5 cc. of the nitroprusside solution; mix thoroughly.

3. Tilt the tube and carefully overlay the mixture with 1 to 2 cc. of a 28 per cent solution of ammonium hydroxide.

4. A purple or purplish-red ring forms at the line of contact in a few minutes if acetone is present. The ring tends to be more purple or violet in low concentrations, more red-purple in high. Amorphous urates may give a brown or orange ring if present in large amount.

**Rantzman's Test.**—1. Prepare the reagent by dissolving 37.5 gms. of ammonium nitrate crystals and 2.5 gms. of sodium nitroprusside in distilled water and make up to 100 cc. In a brownish glass-stoppered bottle it will keep for 2 months.



2. To 3 cc. of urine in a test tube, add 1 cc. of the reagent. Mix and overlay with a 28 per cent solution of ammonium hydroxide.

3. If acetone is present, a sharply defined purple or burgundy-red ring appears at the line of contact. The smaller the amount of acetone, the longer it takes the ring to appear.

**Frommer's Test.**—The color reaction in this test depends upon the formation of dihydroxydibenzoylacetone through the interaction of salicylaldehyde and acetone.

1. To 10 cc. of undistilled urine in a test tube add 3 cc. of 40 per cent solution of sodium hydroxide.

2. Add 10 drops of a 10 per cent alcoholic solution of salicylaldehyde.

3. Mix and heat the upper portion to about 70° C. (do not boil) for 5 to 10 minutes.

4. In the presence of acetone an orange color changing to deep red appears in the heated portion. A yellow to brown color is negative.

### TESTS FOR DIACETIC ACID

**Gerhardt's Test.**—The detection of diacetic (aceto-acetic) acid by this test depends upon the production of a bordeaux red or violet red color with a dilute solution of ferric chloride. *Fresh urine must be used.* A similar color is produced by phenols, coal tar antipyretics, salicylates and other compounds.

1. To 5 cc. of urine in a test tube, add a 10 per cent aqueous solution of ferric chloride, drop by drop, until no further precipitate of phosphates is produced.

2. Filter and add more ferric chloride solution or place a small amount of the reagent in a test tube and carefully overlay with filtrate.

3. The development of a bordeaux red color indicates the presence of diacetic acid or some interfering drug requiring a confirmatory test which is conducted as follows:

4. Place 10 cc. of urine in a beaker, add 10 cc. of distilled water and boil down briskly to 10 cc. Allow to cool and filter if necessary. Repeat the test as above. The absence of a bordeaux red color indicates that the color in the first test was due to diacetic acid which was either boiled off or oxidized to acetone. If the boiled urine gives a bordeaux red color, proceed as follows:

5. Place 10 cc. of urine in a test tube and extract with an equal volume of chloroform. Separate the chloroform and discard. Acidify the urine with 1 cc. of a 10 per cent solution of sulfuric acid. Extract the mixture with an equal volume of ether. Separate the ether and evaporate to dryness in an evaporating dish. Dissolve the residue in 2 cc. of water. Add 2 drops of 10 per cent solution of ferric chloride. A bordeaux red color indicates the presence of diacetic acid.

**Lindemann's Test.**—1. To 10 cc. of urine in a test tube, add 5 drops of 30 per cent acetic acid, 5 drops of Lugol's solution and 3 cc. of chloroform.

2. Shake well and allow chloroform to settle.

3. If diacetic acid is present, the chloroform does not change color but becomes reddish-violet in its absence.

4. If the urine contains much uric acid, use double the amount of Lugol's solution.

### HART'S TEST FOR BETA-OXYBUTYRIC ACID

**Principles.**—Beta-oxybutyric acid is seldom or never present in urine without acetone and diacetic acid. Its presence has the same significance as diacetic acid but

is of more serious import. In this test beta-oxybutyric acid is transformed into acetone which is then detected by treatment with sodium nitroprusside.

**Procedure.**—1. Dilute 20 cc. of urine with an equal amount of water and add a few drops of acetic acid.

2. Reduce to one-half its volume by boiling to remove acetone and diacetic acid.

3. Dilute to 20 cc. with water and place 10 cc. in each of two test tubes.

4. To one tube add 1 cc. of hydrogen peroxide and warm gently for 1 minute. Then allow to cool.

5. To both tubes add 10 drops of glacial acetic acid and 10 drops of freshly prepared concentrated sodium nitroprusside solution.

6. Mix thoroughly.

7. Overlay with strong ammonia water.

8. Allow to stand 3 or 4 hours.

9. A positive result is a purple ring in the tube treated with the peroxide, and none in the other.

### OBERMAYER'S TEST FOR INDICAN

**Principles.**—This test depends upon the decomposition of indican (indoxyl potassium sulfate) and the subsequent oxidation of the liberated indoxyl to indigo blue, at times to indigo red, with absorption by chloroform. Slight traces are present in many normal urines. Bile pigments interfere with the test and must be removed beforehand by adding to the urine one-fifth volume of a 10 per cent solution of calcium or barium chloride and filtering.

**Procedure.**—1. Prepare the reagent by dissolving 2 gms. of ferric chloride in 1000 cc. of concentrated hydrochloric acid (sp. grav. 1.19).

2. To 5 cc. of urine in a test tube, add an equal amount of reagent and 2 cc. of chloroform. Mix by inverting ten times. Allow the chloroform to settle and examine its color.

3. A pale blue to deep blue or violet color indicates the presence of indican, the intensity of the color being proportional to the concentration of indican. If oxidation is slow, a red color due to the formation of indigo red may appear.

4. Iodides may give a red-violet color due to the liberation of iodine. The addition of a few drops of a concentrated solution or a small crystal of sodium thiosulfate will discharge this color. Thymol may produce a violet color which is also discharged by thiosulfate. Urotropin and formalin prevent the appearance of indigo blue even when indican is present in large amounts.

### TESTS FOR BILE PIGMENTS

**Principles.**—Bile pigments give the urine a greenish-yellow, yellow, or brown color, which, upon shaking, is imparted to the foam. Bilirubin is the most important pigment. Upon standing, it may be oxidized to biliverdin. The tests given depend upon the oxidation of bile pigments by acids with the formation of derivatives like biliverdin (green), bilicyanine (blue) and choletelin (yellow).

**Rosenbach's Modification of Gmelin's Test.**—1. Filter 10 to 20 cc. of urine, acidified with 1 or 2 drops of dilute hydrochloric acid, through a small heavy filter paper.

2. Introduce 1 drop of concentrated nitric acid which is slightly yellow (due to the presence of oxides in nitrogen) into the apex of the paper and then unfold it.

3. A positive reaction is indicated by a play of colors appearing in the order of green (biliverdin), blue (bilicyanine), violet, red and reddish yellow (choletelin), the last nearest the center of the paper.

**Hammarsten's Test.**—This is a test for bilirubin, conducted as follows:

1. Prepare a stock reagent by mixing 1 cc. of dilute nitric acid (1 part of concentrated acid diluted with 3 parts of water) with 19 cc. of dilute hydrochloric acid (1 part of concentrated acid diluted with 3 parts of water).

2. Prepare the test reagent by diluting 1 cc. of stock reagent with 4 parts of absolute ethyl alcohol.

3. Place 2 cc. of the test reagent in a test tube and add a few drops of urine.

4. Or, the test may be conducted with a urinary precipitate prepared as follows: To 5 cc. of acid urine (acidify if necessary) add 5 cc. of a 10 per cent solution of barium chloride. Mix well and centrifugalize. Decant and discard the supernatant fluid. Mix the precipitate with 2 cc. of the test reagent and centrifugalize when the reading is made.

5. A positive reaction is indicated by a green color. This test is sensitive to 1 part of pigment in 1,000,000 parts of urine.

**Huppert-Nakayama's Test.**—This is a test for bilirubin, conducted as follows:

1. Prepare the Nakayama reagent by dissolving 0.4 gm. of ferric chloride in a mixture of 99 cc. of 95 per cent ethyl alcohol and 1 cc. of concentrated hydrochloric acid.

2. To 5 cc. of urine in a test tube add 5 cc. of a 5 per cent solution of barium chloride. Mix thoroughly and centrifugalize.

3. Pour off the supernatant fluid. To the sediment add 2 cc. of the Nakayama reagent. Mix and bring to a boil.

4. A brilliant, deep-green color develops if bilirubin is present. On adding a few drops of nitric acid the color changes to violet or red.

### TESTS FOR BILE ACIDS

**Hay's Test.**—In this test advantage is taken of the fact that bile acids have the property of reducing the surface tension of fluids in which they are contained.

1. Cool the urine by placing it in a refrigerator for several hours.

2. Upon the surface sprinkle a little finely powdered sulphur ("flowers of sulphur").

3. If the sulphur sinks at once, bile acids are present to the amount of 0.01 per cent or more. If the sulphur sinks only after gentle agitation, bile acids are present in 0.0025 per cent or more. If the sulphur remains floating, even after gentle shaking, bile acids are absent. Chloroform and turpentine give false positive reactions. Urine preserved with thymol or the presence of large amounts of urobilin may also give falsely positive reactions.

**Pettenkofer's Test.**—This test depends upon the reaction of bile acids and sucrose in acid solution to form a red-colored compound.

1. Place 5 cc. of urine in a test tube and add 5 drops of a 5 per cent solution of sucrose in distilled water.

2. Mix well. Incline the tube and carefully add 3 cc. of concentrated sulfuric acid so that it forms a distinct layer at the bottom of the tube.
3. A red ring at the zone of contact indicates a positive reaction.
4. Mix the two solutions and cool under running water so that the temperature does not rise above 70° C. A positive reaction is indicated by the gradual development of a red color. Proteins and chromogenic substances in the urine may interfere by yielding various colors.

### OLIVER'S TEST FOR BILE SALTS

**Principle.**—This test is based upon the principle that a precipitate is formed of a protein (peptone) and bile acids.

**Procedure.**—1. Prepare the reagent by dissolving 8.33 gms. of peptone and 1.12 gms. of salicylic acid in 1000 cc. of distilled water to which has been added 2 drops of glacial acetic acid.

2. Filter a small amount of urine (5 or 10 cc.) until perfectly clear.
3. Acidify with acetic acid.
4. Dilute with water until specific gravity is less than 1.008.
5. Place 2 cc. in test tube and add 5 cc. of the reagent.
6. A positive reaction is indicated by a milky turbidity which disappears on shaking but reappears when more of the reagent is added. The presence of thymol vitiates the test. A positive reaction may also be obtained in the presence of chloroform or after the administration of turpentine or its derivatives. If the color remains a light red within the time allowed, the reaction indicates normal value. With a little experience one can judge by the color whether there is an increase above normal.

### SCHLESINGER'S TEST FOR UROBILIN

**Principles.**—Urobilin is probably a decomposition product of bilirubin, formed chiefly in the intestines through the action of bacteria. It is now regarded as identical with hydrobilirubin. It is excreted as a chromogen, *urobilinogen*, which is changed into urobilin through the action of light within a few hours after urine is voided. Traces of urobilin, which are too small for detection by ordinary tests, are present in the urine under normal conditions.

1. To 10 cc. of urine in a test tube add a few drops of Lugol's solution.
2. Add 10 cc. of a saturated alcoholic solution of zinc acetate.
3. Mix and filter.
4. View the filtrate in sunlight against a dark background or with light concentrated upon it with a lens; a greenish fluorescence indicates the presence of urobilin.
5. Bile pigment, if present, should be removed previously by adding  $\frac{1}{6}$  volume of 10 per cent solution of calcium chloride and filtering.

### EHRlich's TEST FOR UROBILINOGEN

**Principles.**—As previously stated, urobilinogen is a chromogen derived from urobilin through the action of light within a few hours after the urine is voided. This test, as modified by Wallace and Diamond,<sup>2</sup> is roughly quantitative. Bile pigment, if present, should be removed previously by adding 1 part of a 10 per cent aqueous

solution of calcium chloride to 4 parts of urine, and filtering. The examination of single fresh specimens rather than of 24-hour urine is recommended since at least one specimen with increased urobilinogen may be found during the day, a fact which is significant of liver damage.

**Procedure.**—1. Prepare the reagent by dissolving 2 gms. of paradimethylamino-benzaldehyde in 100 cc. of 20 per cent (by volume) hydrochloric acid.

2. To 10 cc. of bile-free, undiluted urine at room temperature, or warmed to 21° to 22° C., add 1 cc. of the reagent. Allow to stand for 3 minutes.

3. If a deep cherry-red color appears, proceed with the test, using 10 cc. amounts of dilutions of urine prepared with tap water at room temperature as follows: 1:10, 1:20, 1:50, 1:100 and 1:200. Add 1 cc. of reagent to 10 cc. portions of each dilution, let stand 3 to 5 minutes, not longer, and read.

4. Express the results in terms of the highest dilution giving a faint but definite pink or cherry color. Normally, this is at the 1:20 dilution. Any greater dilution yielding a definite pink color indicates a pathological amount of urobilinogen. A daily estimation showing positive in greater and greater dilution is especially significant.

5. When possible, the readings should be made by daylight as artificial light has a tendency to intensify the color. They should be made by viewing the contents through the mouth of the tube, holding it over white paper. Highly concentrated urines may give a yellowish-brown discoloration which has to be differentiated from the true pink reaction of urobilinogen. Pyridium, if present, will give a positive reaction to the test. Therefore, specimens containing this drug are unsuitable for testing. This false positive reaction may be detected by treating the urine with HCl alone. A pink or red color with acid indicates the presence of the dye. By discontinuing the pyridium for 3 or 4 days a true test for urobilinogen can be obtained. Schlesinger's test for urobilin is not affected by pyridium and may be substituted in such cases.

### EHRLICH'S DIAZO TEST

**Principles.**—The exact nature of the diazo substance or substances is unknown. It may be due to an increased excretion of urochromogen, alloxypoteic acid, oxyproteic acid, or uroferic acid. The reaction occurs in the urine in febrile disorders, especially typhoid fever, tuberculosis, and measles. Reactions more or less resembling it may occur after the administration of opium and its alkaloids, salol, creosote, phenol, the iodides, naphthalin and tannic acid.

**Procedure.**—1. Prepare reagent No. 1 by dissolving 1 gm. of sulfanilic acid in 200 cc. of water and 10 cc. of concentrated hydrochloric acid.

2. Prepare reagent No. 2 by dissolving 0.5 gm. of sodium nitrite in 100 cc. of water.

3. In a test tube, mix 10 cc. of reagent No. 1 with 0.1 cc. of reagent No. 2.

4. Add an equal volume of urine. Mix and overlay with 2 cc. of 28 per cent solution of ammonium hydroxide.

5. A positive reaction is indicated by the development of eosin pink to deep crimson red color at the line of contact. Upon shaking, a distinct pink color is imparted to the foam (essential feature). The color is a pure pink or red; any trace of yellow or orange is a negative reaction. A doubtful reaction should be considered negative.

## TESTS FOR PORPHYRINS

**Principles.**—Porphyrins are substituted tetrapyril methenes. Small amounts may be found in normal urine. Urine containing large amounts (*porphyria*) may be dark red or a "port wine" color. Darkening of a light colored urine upon standing 24 hours or longer is suggestive of porphyria.

**Procedures.**—1. To 25 cc. of urine in a separatory funnel, add 10 cc. of glacial acetic acid. Extract this mixture twice with 50 cc. portions of ether and combine the extracts. Wash the combined extracts with 10 cc. of 5 per cent hydrochloric acid.

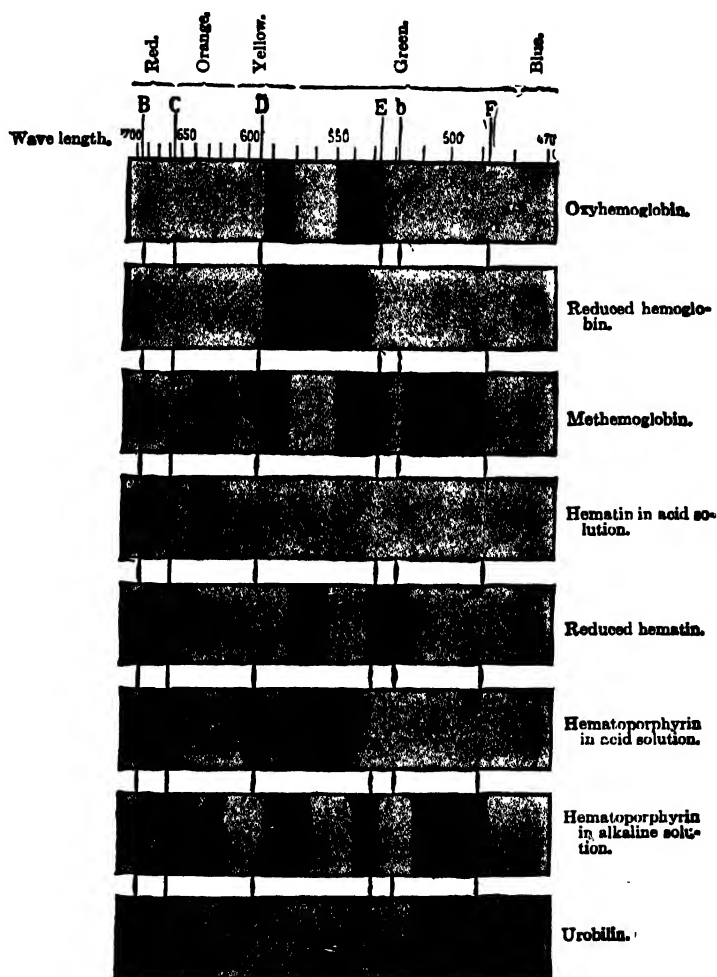


FIG. 85.—ABSORPTION SPECTRA  
(After Seifert and Müller.)

2. Examine the washings under ultraviolet light. If there is a strong red fluorescence, a large amount of coproporphyrin is present, suggestive of porphyria. Examine the urine residue after ether extraction under ultraviolet light. A discernible red

fluorescence is indicative of the presence of uroporphyrin. These tests should be confirmed spectroscopically as follows:

3. To 100 cc. of urine add 20 cc. of a 10 per cent solution of sodium hydroxide.
4. Filter or centrifugalize off the precipitate.
5. Wash the precipitate with water and with alcohol.
6. Add 5 cc. of alcohol and 5 to 10 drops of concentrated hydrochloric acid.
7. Dissolve, filter until absolutely clear, and examine spectroscopically for the absorption bands of acid hematoporphyrin (Fig. 85).
8. An acetic acid test, which is much less reliable, consists in adding 5 cc. of glacial acetic acid to 100 cc. of urine and allowing the mixture to stand 48 hours. The pigment deposits in the form of a precipitate.

### TESTS FOR MELANIN

**Principles.**—Urine which contains melanin (*melanuria*) darkens upon exposure to air, assuming a dark-brown or black color, due to the fact that melanin is eliminated as a chromogen-melanogen which is later converted into the pigment. Melanuria is probably due to protein destruction which may occur in widely different diseases but especially in melanotic tumors.

**Procedures.**—1. Add a few drops of a solution of ferric chloride to 10 cc. of urine. If melanin is present, a gray precipitate forms which blackens on standing.

2. Mix equal parts of urine and bromine water. If melanin is present a yellowish precipitate forms which gradually turns black.

A better test, described by Blackberg and Wanger,<sup>3</sup> is as follows:

1. Evaporate a 24-hour specimen of urine to  $\frac{1}{3}$  of its original volume and, for each 100 cc. of concentrated urine, add 1 gm. of potassium persulphate.

2. Allow to stand 2 hours and add an equal volume of absolute methyl alcohol; mix and allow the precipitated melanin to settle.

3. Filter off the precipitate, wash with water until the washings are colorless, and then wash with methyl alcohol to remove any remaining soluble pigments.

4. Finally, wash with ether.

5. If melanin is present, a brownish-black precipitate remains on the filter. The precipitate is soluble in 5 per cent sodium hydroxide solution from which it may again be precipitated by the addition of acid.

### TESTS FOR CHLORIDES

The following simple qualitative test will show the presence of chlorides (mainly in the form of sodium chloride) and at the same time roughly indicate any pronounced alterations in the amount:

1. Place 5 cc. of albumin-free urine in a test tube and add 3 drops of nitric acid to prevent the precipitation of phosphates.

2. Add 5 drops of a 12 per cent solution of silver nitrate in distilled water. Normally a white curdy precipitate of silver chloride forms. If the urine merely becomes milky or opalescent, chlorides are markedly diminished.

The McLean and Selling<sup>4</sup> modification of the Volhard method is preferred. As a rule, albumin need not be removed. The technic is as follows:

1. Prepare reagent No. 1 by dissolving 29.055 gms. of C.P. anhydrous, crystallized silver nitrate in 900 cc. of a 25 per cent solution of nitric acid. Add 50 cc. of a cold saturated solution of ammonioferric alum and distilled water to 1000 cc.

2. Prepare reagent No. 2 by dissolving 7 gms. of ammonium sulfocyanate in 1000 cc. of distilled water. This solution is intentionally made too strong, and must be standardized by diluting with distilled water until exactly 20 cc. (no less) will produce a red color when mixed with exactly 10 cc. of reagent No. 1.

3. Place 5 cc. of urine and 10 cc. of reagent No. 1 in a *certified* 50 cc. graduated cylinder with a glass stopper. Mix by inverting several times.

4. If a reddish color develops, add 3 drops of a 10 per cent solution of potassium permanganate in distilled water.

5. After 5 minutes add reagent No. 2, a very little at a time, mixing after each addition until a permanent reddish-brown color (best seen against a white background) appears. This is the end-point.

6. The solutions are so balanced that if the urine is chloride-free the volume of fluid when the end-point is reached will be 35 cc. and that for each gram per liter of chlorides in the urine will be 1 cc. less. Therefore, the difference between 35 cc. and the height of the fluid at the end of the test gives directly the number of grams of chlorides per liter of urine, expressed as sodium chloride. If, for example, the fluid reaches the 30-cc. mark,  $35 - 30 = 5$  gm. of sodium chloride per 1000 cc. of urine. If the total 24-hour output of urine is known the total amount of sodium chloride may be calculated. Normally this varies from 10 to 16 gms. per 24-hour urine.

### SULKOWITCH TEST FOR CALCIUM

This is a very simple roughly quantitative test for urinary calcium,<sup>5</sup> the technic of which is as follows:

1. The patient should be kept on a neutral, low-calcium diet before the test is conducted. A 24-hour specimen of urine is preferred.

2. Prepare the reagent by dissolving 2.5 gms. of oxalic acid crystals and 2.5 gms. of ammonium oxalate crystals in 145 cc. of distilled water; and 5 cc. of glacial acetic acid.

3. In a test tube mix equal parts of clear urine and reagent.

4. If there is no precipitate, the total serum calcium is probably reduced to between 5 to 7.5 mg. per 100 cc. A fine white precipitate indicates a normal range of serum calcium (9 to 11 mg.). A milky precipitate indicates an increase of calcium (may be observed after the ingestion of calcium in powder or large amounts of milk).

### SACCHAROGENIC TEST FOR URINARY AMYLASE

This test, which is the Dozzi modification of the Somogyi method, is conducted as follows:

**Preparation of Starch Paste (Small).**—Weigh out approximately 100 gm. of starch and transfer to a 1000 cc. Erlenmeyer flask; add 500 cc. of 95 per cent alcohol and 4 cc. of concentrated hydrochloric acid. The flask is then fitted with a reflux condenser, placed in a boiling water bath for 15 minutes and vigorously shaken from time to time to keep the starch from settling out. At the end of this interval it is



removed, the acid exactly neutralized with a normal solution of sodium hydrogen carbonate and the alcohol filtered off as quickly as possible. The soluble starch is washed with distilled water to free it from the acid. The product thus obtained is dried at room temperature, sieved and preserved as a stock sample.

**Preparation of Buffer Solution (Schmidt, Greengard and Ivy).**—Prepare the buffered starch solution by boiling 10 gm. of soluble starch with 250 cc. of distilled water containing 2.92 gm. NaCl (0.2 M solution). Add 250 cc. of phosphate buffer solution (70 cc. of 0.2 M  $\text{KH}_2\text{PO}_4$  and 180 cc. of 0.2 M  $\text{Na}_2\text{HPO}_4$ ). The final pH of the 2 per cent starch-phosphate mixture should be 6.8.

**Procedure.**—1. The urine is filtered and placed in either a water bath or incubator at 40° C. In the meantime a fresh 2 per cent soluble starch solution is prepared and buffered to a pH of 6.8 with a 0.2 M phosphate and 0.2 M NaCl solution as described above.

2. Ten cc. of buffered starch solution are pipetted into each of two 250 cc. Erlenmeyer flasks. Contamination with saliva must be avoided. One flask is placed either in a constant temperature water bath or in an incubator at 40° C. and allowed to remain until the flask and contents have assumed a temperature of 40°. During this time 10 cc. of urine are added to the other flask followed immediately by 15 cc. of West's  $\text{HgSO}_4\text{-H}_2\text{SO}_4$  mixture (*Biochem. Jour.* 26: 1720, 1932) which serves as a blank.

3. When the flask and contents in the incubator have assumed the desired temperature, 10 cc. of urine are added and allowed to digest for exactly 15 minutes. (It is possible that when dealing with samples of urine that exhibit very slight amylolytic activity, one can increase the efficiency of the test either by using a larger sample of urine or by allowing the incubation to proceed for a longer period of time.) At the end of this time the enzymatic activity is interrupted by the addition of 15 cc. of West's  $\text{HgSO}_4\text{-H}_2\text{SO}_4$  mixture. A pinch of Lloyd's reagent is then added to each flask, and thorough shaking is instituted.

4. To each flask 35 gm. of  $\text{BaCO}_3$  are added and then 50 cc. of distilled water. The mixture is stoppered and agitated until  $\text{CO}_2$  ceases to be produced. The material is now filtered and the filtrate acidified by the addition of 1 drop of concentrated  $\text{H}_2\text{SO}_4$ . The mercury is eliminated by saturating the filtrate with  $\text{H}_2\text{S}$ , and the excess  $\text{H}_2\text{S}$  is driven off by bubbling air through the solution. The solution is filtered and the filtrate rendered alkaline by the addition of a few crystals of  $\text{Na}_2\text{CO}_3$ , phenol red being employed as the indicator.

5. The amount of reducing material in 5 cc. aliquots (a smaller quantity of the filtrate should be used if large amounts of reducing materials are present) of the slightly alkaline filtrate is determined according to the Shaffer-Hartmann method. The quantity of reducing sugar formed by the enzymatic action is in turn calculated by subtracting the amount of reducing material in the blank from that of the digested specimen. For convenience the end result is expressed in terms of glucose.

6. The normal range of amylase expressed in terms of milligrams of glucose varies from 264 to 953. The method is not satisfactory when there is marked glycosuria.

**FABRICUS-MOLLER URINE TEST FOR AMYLASE**

1. A sample of 24-hour specimen of urine is preferred.
2. Prepare a starch solution by dissolving 0.1 gm. of soluble starch in 100 cc. of buffer solution with a pH of 6.7. Add 0.45 gm. of sodium chloride. The starch should be stirred into the cold buffer solution and then dissolved by gentle heating. The solution should be freshly prepared.
3. Dilute 1 cc. of urine with 29 cc. of distilled water.
4. Place 6 test tubes in a rack. Add 2 cc. of starch solution to each. Add 8 drops of diluted urine to No. 1, 6 drops to No. 2, 4 drops to No. 3, 3 drops to No. 4, 2 drops to No. 5, and 1 drop to No. 6.
5. Mix and place the tubes in a water bath at 30° C. to 40° C. for 30 minutes.
6. Cool the tubes in ice water to stop enzyme activity.
7. Add 1 drop of iodine indicator ( a 1:5 dilution of Gram's iodine solution) to each tube, or add 1 drop of N/200 iodine solution.
8. The end-point of the reaction is marked by no color or by a port wine color, a blue color indicating the absence of amylase.
9. Normally, digestion occurs in tube No. 1 (150 or less units), No. 2 (200 units) and tube No. 3 (300 units) by this method. An increase of amylase is indicated by digestion in No. 4 (400 units), No. 5 (600 units) or No. 6 (1200 units).

**BENZIDINE TEST FOR OCCULT BLOOD**

This test is very sensitive provided the reagents are satisfactory. Different lots of benzidine vary greatly in sensitivity and hydrogen peroxide solution rapidly deteriorates. For this reason it is always advisable to set up a positive control using water with an extremely small amount of blood added.

1. Prepare a saturated solution of benzidine crystals in glacial acetic acid. If kept in a brown bottle in a dark place, this solution will keep fairly well. Or, prepare the reagent as required by dissolving as many crystals as can be picked up on the point of a knife in 5 cc. of glacial acetic acid and warm gently to effect solution.

2. The usual solution of hydrogen peroxide is 3 per cent. Test before use by adding a few drops of concentrated sulfuric acid. If the peroxide is still active, a blue color will develop.

3. To 2 cc. of urine, or to the centrifugalized sediment mixed with 2 cc. of water, add 3 cc. of the benzidine solution and 1 cc. of hydrogen peroxide.

4. A positive reaction is indicated by the development of a green to deep blue color. A confirmatory test may be conducted by adding a drop or two of glacial acetic acid to 10 cc. of urine. Extract with 5 cc. of ether. Evaporate the ether extract to dryness, using a water bath which has been heated to boiling and the flame then turned off. Add 1 cc. of water and stir to dissolve the residue—then add a few drops of benzidine solution and a drop or two of hydrogen peroxide.

**MICROSCOPIC EXAMINATION OF SEDIMENTS**

**General Technic.**—1. As far as possible, specimens should be examined within 6 hours after voidance. Unless kept at a low temperature, 24-hour specimens should have a preservative added. Alkaline specimens cloudy with phosphates and obscuring

other elements may be slightly acidified with dilute acetic acid to re-dissolve them. Highly acid specimens containing heavy sediments of urates obscuring other elements, may be slightly warmed to re-dissolve them.

2. Secure sediment by centrifuging at least 15 cc. for 3 to 5 minutes or by allowing the urine to stand at least 6 to 12 hours in a cool place for settling by gravity (preferably in a conical container).

3. Remove a drop of sediment by means of a pipet and place on a slide. The pipet may be a piece of tubing drawn to a blunt point and fitted with a nipple. Eight sediments may be prepared at one time by using slides of ordinary window glass, 4 by 8 inches, divided by painted lines into 8 compartments. The stage of the microscope may be extended by a wooden table, but this is not absolutely necessary.

4. Coverglasses are not essential for ordinary examination but are advisable for high-power examinations.

5. The examination must be completed before drying takes place.

6. Examine with low power and with oblique illumination obtained by swinging the mirror a little out of the optical axis. *Too strong illumination and too great magnification are common sources of error.*

7. The frequency of occurrence of the various constituents observed should be noted as well as their mere presence. The terminology used may be: occasional, few, many, very many, etc. A uniform technic of examination and of reporting should be followed so that the results of different examinations may be uniform and comparable. The same amount of urine should be centrifugalized at the same speed for the same length of time in each case. The supernatant urine should be poured off to the same degree of completeness, and approximately the same thickness of drop examined.

**Casts.**—Casts vary greatly in size but in almost all instances their sides are parallel and ends rounded or broken off squarely. They may be straight or curved, long or short, but the diameter is usually uniform throughout the length. Casts have been classified according to their microscopical characteristics as hyaline, granular, epithelial, blood, pus, fatty, and waxy. The finding of casts in the urine is very important, for their presence usually indicates some form of kidney disorder, especially if albumin is also present.

**Hyaline Casts.**—These casts are colorless, homogeneous, semi-transparent and cylindrical in shape (Fig. 86). They are difficult to see unless the proper illumination is used. Although described as homogeneous, upon careful examination a small amount of granular material is usually seen adhering to them. They are usually straight with parallel sides and rounded ends. Occasionally, curved and convoluted forms are seen. They are readily soluble in acetic acid in contrast to fatty casts which are insoluble.

**Granular Casts.**—These are hyaline casts containing many fine or coarse granules. Those having very fine granules are called "*finely granular*" and those having coarse granules, "*coarsely granular*" (Fig. 86).

**Waxy Casts.**—These are homogeneous like the hyaline cast, but more opaque. They have a dull waxy appearance and are often grayish. All gradations between hyaline and waxy casts may be found. No doubt the more refractive hyaline casts are often incorrectly reported as waxy (Fig. 86).

**Fibrinous Casts.**—These are similar to waxy casts except for their color, which is distinctly yellowish. The color is probably due to altered blood pigment.

**Fatty Casts.**—These are casts of any kind which contain numerous fat droplets.

The fatty nature of the globules can be easily determined by staining with osmic acid or sudan 111. They are insoluble in acetic acid. The source of these fat droplets is chiefly from degenerated epithelial cells (Fig. 86).



FIG. 86.—URINARY CASTS

1. Hyaline casts (after Riedert). 2. Hyaline and finely granular casts (after Todd and Sanford). 3. Waxy and granular casts (after Riedert). 4. Granular and fatty casts (after Riedert).

**Epithelial Casts.**—These contain epithelial cells from the renal tubules. Dilute acetic acid brings out the nuclei more distinctly and aids in the recognition of the cells (Fig. 87). These casts are rare.

**Pus Casts.**—These are composed entirely of pus cells. Finding an occasional pus cell in casts has no special significance. It is only when the cast is nearly or completely filled with pus cells that they are reported as pus casts.

**Blood Casts.**—These contain many erythrocytes which are often degenerated (Fig. 88).

**Pseudocasts.**—*Mucous threads* often appear as long strands resembling hyaline casts. They are more ribbon-like, tapering at the ends and have less defined edges (Fig. 87).

*Urates or phosphates* may aggregate into cylindrical masses resembling granular casts. The application of heat will dissolve the urates, and acetic acid the phosphates.

**Cylindroids.**—These are formations which closely resemble hyaline casts. They are, however, longer, more ribbon- or band-like and taper to a slender tail which is often twisted or curled (Fig. 87).

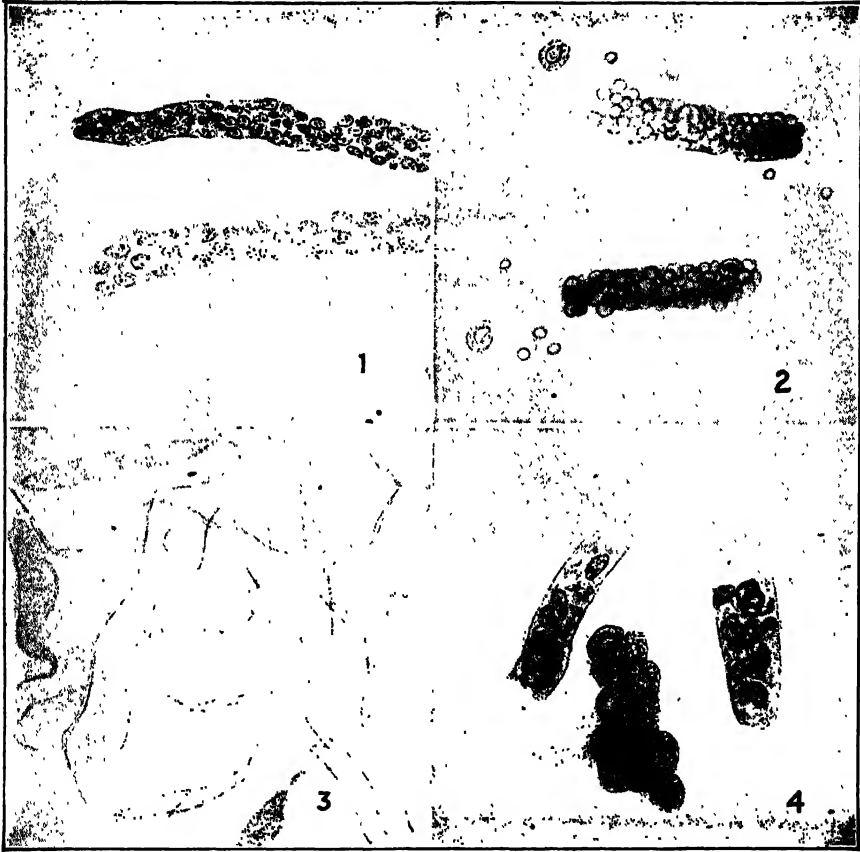


FIG. 87.—URINARY CASTS AND PSEUDOCASTS

1. Epithelial casts (after Riedert). 2. Blood casts (after Todd and Sanford). 3. Mucous threads and cylindroids (after Todd and Sanford). 4. Pseudocasts composed of swollen epithelial cells (after Riedert).

**Leukocytes and Pus.**—A small number of leukocytes are present in normal urine. Any marked increase in their number is significant of disease somewhere along the urinary tract in males, and from either the urinary or genital tracts in the female, unless the specimen is obtained by catheterization. Contamination of the specimen with vaginal discharge may introduce large numbers of leukocytes. There is no way of determining whether a leukocyte is dead or alive except in the case of those showing degenerative changes such as swelling, disintegration and a tendency to aggregate in clumps. It is common practice to call them leukocytes when they occur in normal numbers and *pus cells* when they are definitely increased. The nuclei are frequently

indistinct or obscured by granules. The addition of a little dilute acetic acid brings the nuclei clearly into view (Fig. 89).

Pus, when at all abundant, adds an appreciable amount of albumin and forms a white sediment resembling that produced by phosphates. The leukocytes usually found in urine are neutrophils (polymorphonuclear). Occasionally plasma cells are present, usually with neutrophils. These have a single round nucleus, located at one side of the cell. In alkaline urines the pus may be transformed by the alkalis into a gelatinous substance giving the urine a mucilaginous consistency.

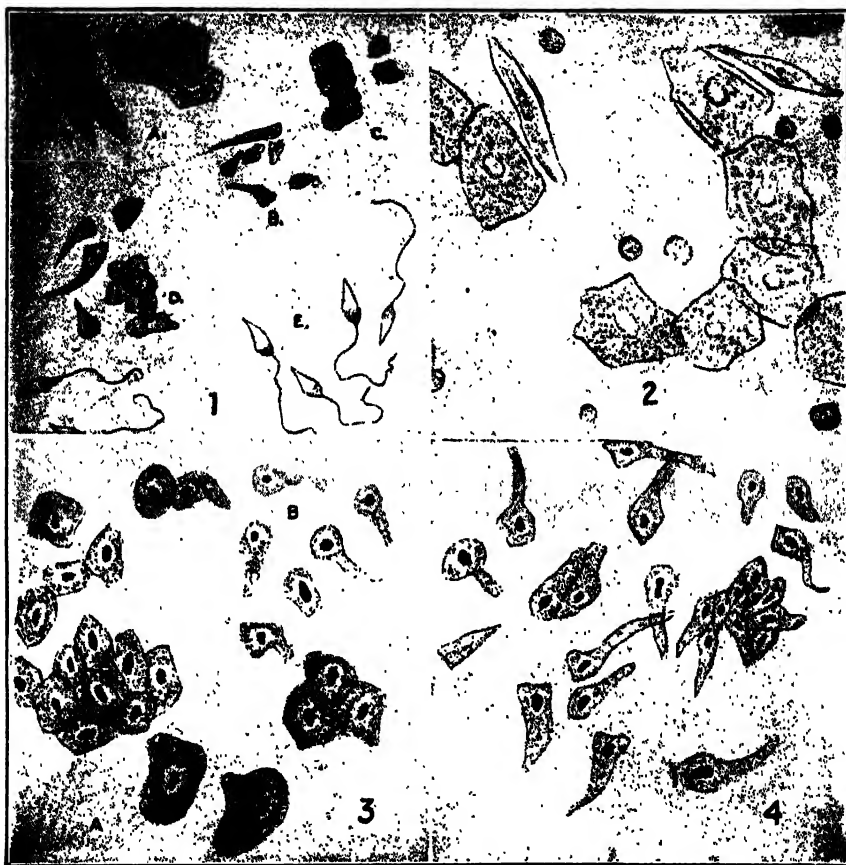


FIG. 88.—URINARY EPITHELIUM AND SPERMATOZOA

1. A, vaginal cells; B, urethral cells; C, renal cells; D, cells from the pelvis of kidney; E, spermatozoa (all after Riedert).
2. Squamous and pus cells (after Todd and Sanford).
3. A, bladder cells; B, urethral cells (after Todd and Sanford).
4. Cells from pelvis of kidney (after Todd and Sanford).

**Erythrocytes.**—The presence of large numbers of erythrocytes is always pathological when contamination with menstrual discharge can be excluded. The cells may have a normal appearance or may be crenated, swollen or hemolyzed (Fig. 89). When blood is present in a large amount, it will change the color of the urine to a hazy reddish or brown color, commonly called "smoky".

When the cells are atypical or there is any doubt, add a little dilute acetic acid.

If they are erythrocytes they will dissolve and disappear. Occult blood tests can be used as confirming tests. However, they are not sensitive enough in cases where only a few cells are found microscopically.

**Epithelial Cells.**—These may be of various kinds as follows:

**Squamous or Pavement.**—These are large flat cells with a small distinct round or oval nucleus (Fig. 88). They come from ureters, bladder or the genitalia; as a rule, except in specimens from the female, they are present in relatively small numbers. Specimens from the female are commonly contaminated with cells of this type originating from the vagina. Although the vaginal cells are especially large, thin, angular and frequently rolled up, it is very difficult to determine the source of these cells in such cases with any degree of certainty.

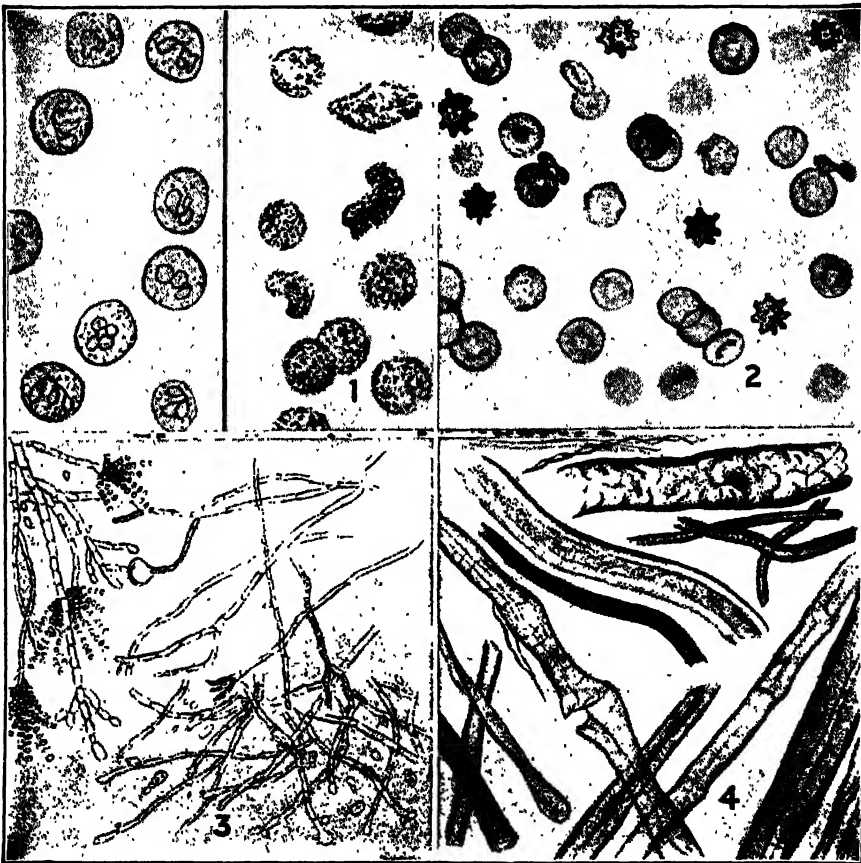


FIG. 89.—URINARY LEUKOCYTES, ERYTHROCYTES, MOLDS AND ARTEFACTS

1. Leukocytes (after Todd and Sanford). 2. Erythrocytes (after Todd and Sanford).
3. Molds (after Riedert). 4. Artefacts (after Riedert).

**Small Round or Polyhedral.**—These cells are somewhat larger than the polymorphonuclear leukocyte and have a single round nucleus. They come from the uriniferous tubules or the deeper layers of any part of the urinary tract. Only when these cells are found adhering to casts can they be considered as of renal origin (Fig. 88).

**Caudate.**—These are smaller than the squamous cell and have tail-like processes.

They come most frequently from the pelvis of the kidney but may also originate from the neck of the bladder (Fig. 88).

**Spermatozoa.**—These are usually present in specimens collected after nocturnal emissions or prostatic massage and as the result of vaginal contamination after coitus. They are easily recognized (Fig. 88).

**Bacteria.**—Normal urine does not contain bacteria. However, urine is readily contaminated when passing through the urethra and with vaginal secretions in the female. Since urine is a very good culture medium for many bacteria, multiplication takes place rapidly. Bacteria, when in large numbers, cause uniform cloudiness of the specimen which will not clear by filtration. In disease, pathogenic bacteria may be present, which require special examination for their detection.

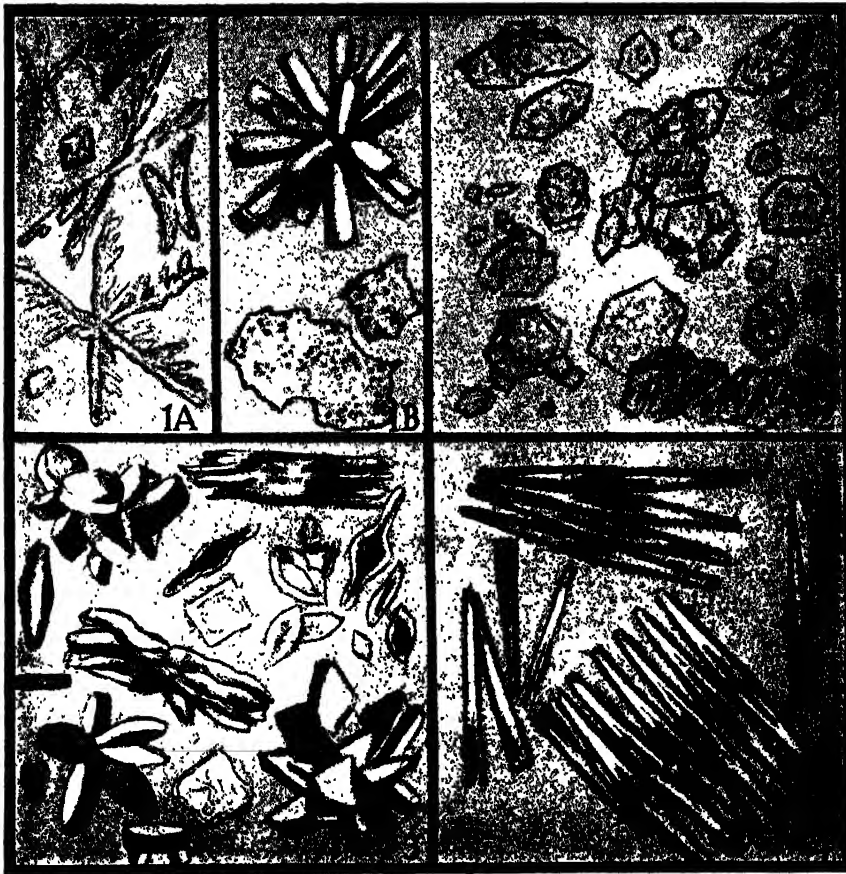


FIG. 90.—URINARY CRYSTALS AND DIATOMS

1A, triple phosphates (feathery type); 1B, calcium phosphate; 2, cystine; 3, uric acid crystals; 4, diatoms.

**Crystals.**—Those which may occur in *acid urine* comprise uric acid and urates, calcium oxalate, cystine, leucine and tyrosine. Those which may occur in *alkaline urine* comprise acid ammonio-magnesium phosphate ("triple phosphates"), amorphous phosphates, ammonium biurate and calcium carbonate. *All of the crystals found in alkaline*



*urines are soluble in acetic acid.* Any of the crystals found in acid or alkaline urine may occur in *neutral urine*. In addition, dicalcium phosphate may be present.

*Uric Acid and Urates.*—These do not appear normally in urine at the time of voidance. All acid urines upon standing, particularly when cold, will precipitate the uric acid normally present in solution in the form of crystals or as amorphous urates. *Therefore, the finding of uric acid crystals in other than freshly voided specimens is of*

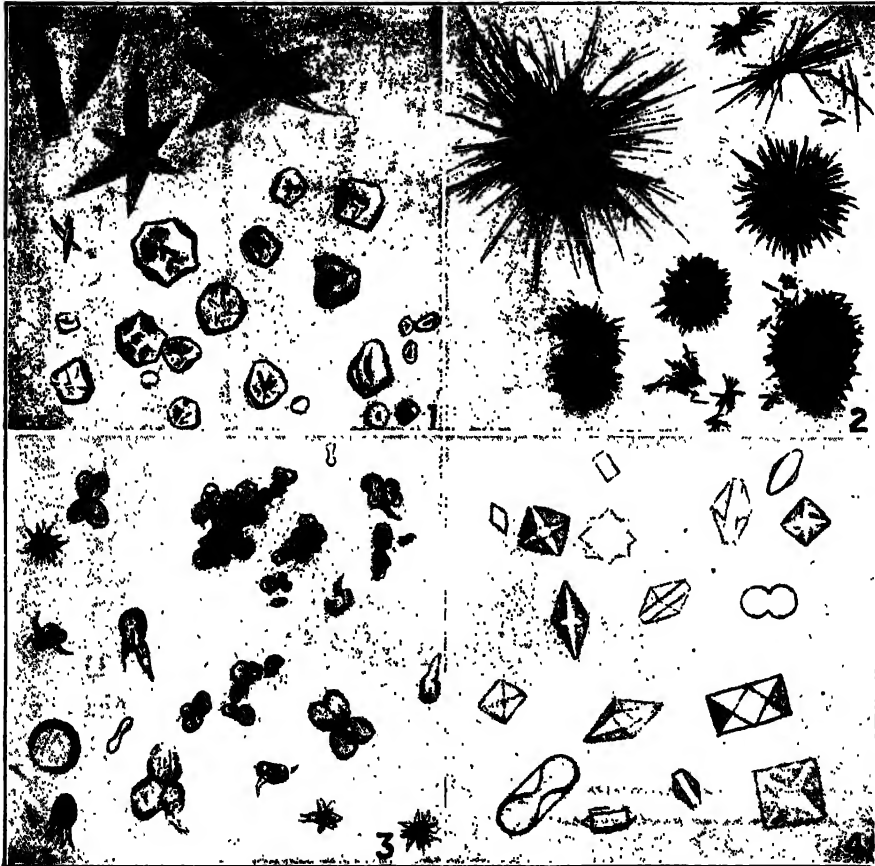


FIG. 91.—URINARY CRYSTALS

1. Uric acid (after Riedert). 2, Calcium urate (after Riedert). 3. Acid ammonium urate (after Riedert). 4. Calcium oxalate (after Todd and Sanford).

*no clinical significance.* The urates produce cloudiness and in highly concentrated specimens may appear milky or have a pinkish or reddish color. The former may be mistaken for pus and the latter for blood. A simple test is to warm the specimen, whereupon the urates will redissolve and the specimen will become cleared of this precipitate. The crystals may settle out and appear as red grains (gravel or red sand). Microscopically they vary greatly in size and shape (Figs. 90 and 91). The typical ones have a yellow or reddish brown color. This color is due to urinary pigment, chiefly uro-erythrine. Colorless crystals are sometimes seen. If these are hexagonal, they may resemble cystine. All colored crystals found in acid urine can safely be considered as

uric acid irrespective of their shape. They are soluble in sodium hydroxide, insoluble in hydrochloride or acetic acid, and dissolve in ammonia with the formation of crystals of ammonium urate.

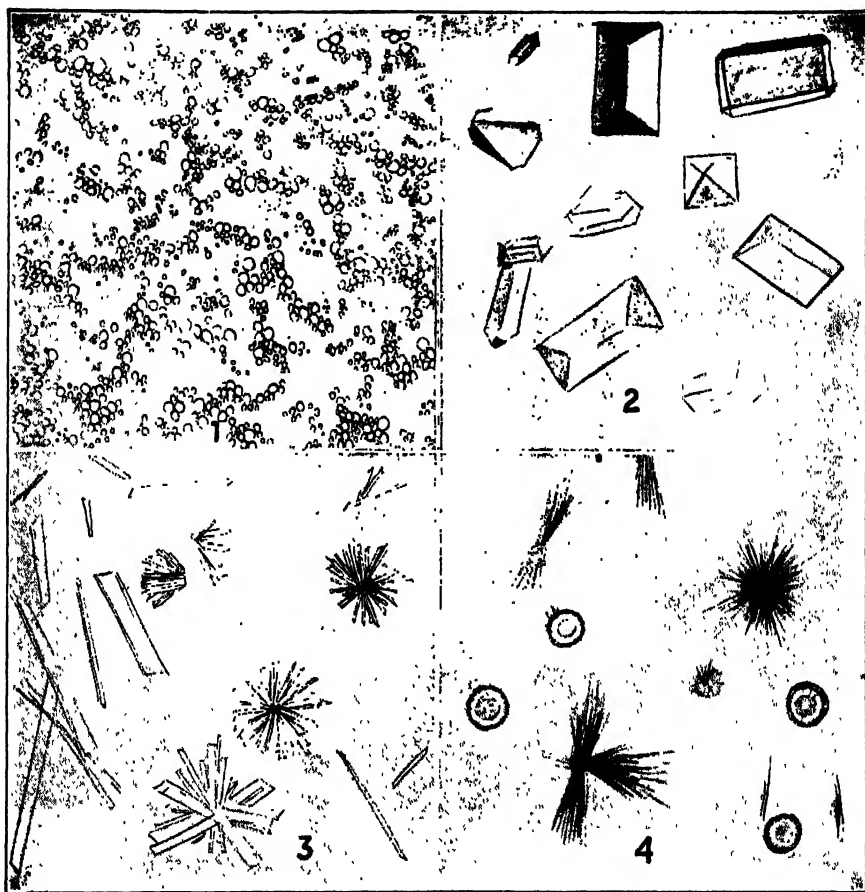


FIG. 92.—URINARY CRYSTALS

1. Amorphous phosphates (after Riedert). 2. "Triple phosphates" (after Todd and Sanford). 3. Calcium sulphate (after Riedert). 4. Leucine and tyrosine (after Riedert).

**Calcium Oxalate.**—These crystals, commonly found in acid urine, may occasionally be seen in neutral or slightly alkaline urine. Their presence is of clinical significance only when found immediately after voidance. The common cause of their presence is the ingestion of foods which are rich in oxalic acid, such as tomatoes, spinach, rhubarb and asparagus. They usually appear as colorless, octahedral crystals, having the appearance of small squares crossed by two intersecting diagonal lines, the so-called "envelope crystals". They vary greatly in size and may occur as dumb-bells or spheres (Fig. 91). They are soluble in strong hydrochloric acid, and recrystallize upon the addition of ammonia.

**Cystine.**—Cystine is one of the amino acids formed in the decomposition of protein. It is present in traces in normal urine but only when an excessive amount is present are crystals formed. They are rarely seen. They occur as colorless, highly refractive,

hexagonal plates with well defined edges, and are soluble in hydrochloric acid and insoluble in acetic acid (Fig. 90).

*Leucine and Tyrosine.*—These substances rarely occur in urine. Both are cleavage products of the protein molecule and usually occur together. *Leucine* appears in the forms of yellowish, oily looking spheres, many with radial and concentric striations (Fig. 92). It is not soluble in hydrochloric acid or in ether. *Tyrosine* appears as very fine needles which may appear black and are usually arranged in sheaves, with a con-

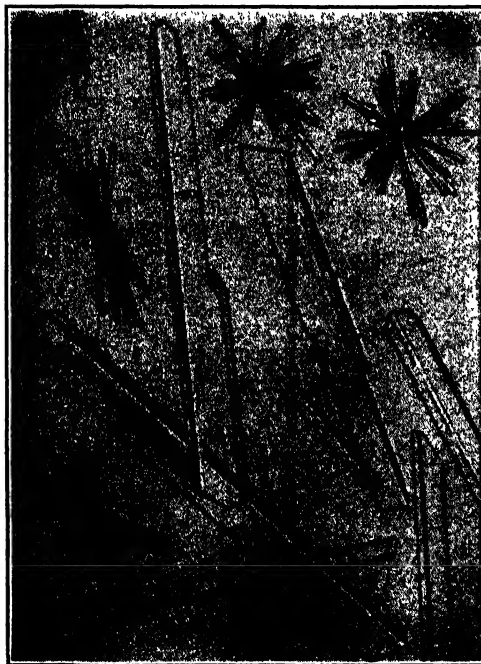


FIGURE 93

FIG. 93.—CRYSTALS OF SULFANILAMIDE

Free sulfanilamide appears as long transparent crystals with a tendency to aggregate. Acetylsulfanilamide forms sheaves and radiating bundles of prismatic crystals. (Courtesy of the *Seminar*, published by Sharp and Dohme, Inc.)

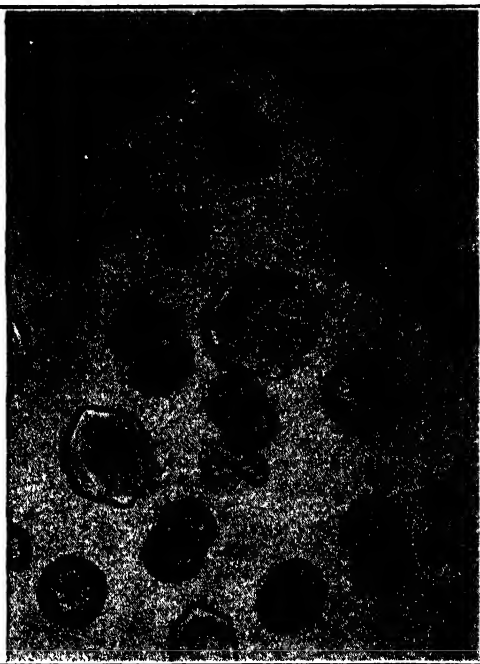


FIGURE 94

FIG. 94.—CRYSTALS OF SULFATHIAZOLE

These crystals have the appearance of shocks of wheat with a central binding. These may present all degrees of fullness up to a stage where they form two half circles fused at the center. They may also form complete rosettes. Another form is that of hexagonal platelets of variable density which may be indented at ends and show an envelope-like marking. (Courtesy of the *Seminar*, published by Sharp and Dohme, Inc.)

striction in the middle (Fig. 92). It is soluble in ammonia and hydrochloric acid, but not in acetic acid. Leucine and tyrosine crystals are difficult to recognize by their morphology as other substances may take similar or identical forms.

*Dicalcium phosphate* may be found in the form of colorless prisms arranged in stars or rosettes in feebly acid, neutral or feebly alkaline urines (Fig. 90).

*Ammonio-magnesium Phosphate ("Triple Phosphates").*—These crystals are commonly found in alkaline urine. The typical shape is a prism with oblique ends often

called the "coffin-lid" form. They may, when rapidly precipitated, take feathery or leaf-life forms (Figs. 90 and 92).

**Amorphous Phosphates.**—Common in alkaline urine and appear as a granular precipitate (Fig. 92).

**Acid Ammonium Biurate.**—It is precipitated only when free ammonia is present and is therefore usually found along with phosphates in decomposing urine. It occurs in the form of yellow spheres with a spicule, the so-called "thorn-apple crystals". Occasionally they form sheaves of fine needles and rhizome forms.

**Calcium Carbonate.**—Occurs as amorphous granules or colorless spheres and dumb-bells. It dissolves readily in acetic acid with the formation of gas.

**Sulfonamide Crystals.**—When microscopic or macroscopic hematuria occurs during treatment with the sulfonamide compounds it is important to examine the urine for the crystals of these compounds. The latter usually occur as crystals of free and acetylated compounds, especially the latter. *Fresh urine should be employed*, sediment being secured by centrifugalization. The crystals produced by sulfanilamide, sulfathiazole, sulfapyridine and sulfadiazine are shown in Figures 93, 94, 94A and 94B.

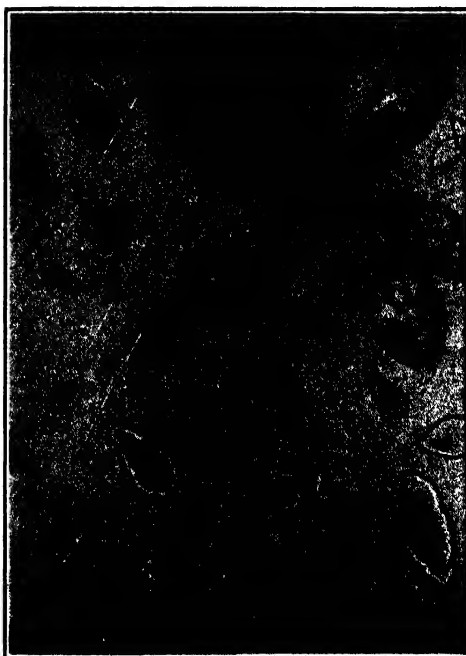


FIGURE 94A

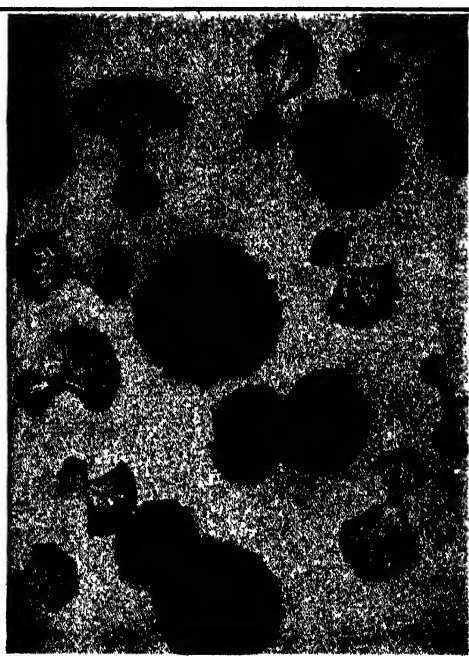


FIGURE 94B

FIG. 94A.—CRYSTALS OF SULFAPYRIDINE

Sulfapyridine commonly appears in the urine as acetylsulfapyridine. The most frequent form is the whetstone, petal or boat-shaped crystals. Jagged arrowheads pointed at one end are also fairly common. Large conglomerate crystals may occur but are not usual. (Courtesy of the *Seminar*, published by Sharp and Dohme, Inc.)

FIG. 94B.—CRYSTALS OF SULFADIAZINE

Free sulfadiazine forms dark, dense, greenish globules with either fuzzy or clean edges. Acetylsulfadiazine crystals form "shocks of wheat" with eccentric binding. The eccentric binding differentiates it from the central binding of acetylsulfathiazole crystals. (Courtesy of the *Seminar*, published by Sharp and Dohme, Inc.)

**Extraneous Matter.**—The accidental contamination of urine with extraneous matter is not at all uncommon. The source is usually the use of unclean containers or utensils in collecting specimens. *Yeasts* are often found in specimens which have stood for some time. They multiply rapidly, particularly in urines containing sugar. They may be mistaken for erythrocytes from which they may be distinguished by their oval shape, tendency to form chains, the presence of budding forms, and their insolubility in acids or alkalis. *Molds* are commonly found in urine which has stood 24 hours or more (Fig. 89). *Fibers* of wool, cotton, linen or silk may come from clothing, towels or the air (Fig. 89). *Oil droplets*, which are highly refractive, may come from oiled catheters or unclean containers. *Diatoms* from tap water are not uncommon (Fig. 90).

### ADDIS SEDIMENT COUNT

**Principles.**—This test is based upon the principle that the number of casts and cells in the urine varies with the type and extent of kidney disease. It is of the utmost importance that the patient clearly understand the conditions under which the specimen must be secured, and it is advisable that, in order to prevent errors, the directions be furnished in writing.

In order that the specimen may reach the laboratory in a suitable condition, the urine should be passed directly into a wide-necked bottle which has been thoroughly cleansed, rinsed with distilled water, dried in the inverted position, and finally rinsed out with a clean solution of formaldehyde, allowed to drain out for a short time before the bottle is closed with a rubber stopper. In women, catheterization is essential and it is important that the bladder be emptied completely.

**Procedure.**—1. The patient is instructed to take breakfast as usual, including coffee, tea or milk, as desired, but *must abstain from all fluids thereafter* during the day and night until the urine has been collected in the morning. In other respects, the diet is unaltered, except that specific instructions are given not to take more fruit than is customary. The time of collection may run from any convenient hour in the evening until the patient arises in the morning, covering at least 12 hours. The patient is asked not to void during the afternoon of the day on which the collection is begun. The first voiding is discarded, all others being passed directly into the container as above described, care being taken to empty the bladder as completely as possible. The time when the collection is begun and ended must be recorded on the bottle.

2. Any uratic precipitate is dissolved by immersing the bottle in warm water; phosphatic turbidity is cleared by the addition of acetic acid, avoiding an excess.

3. Measure the volume (to within  $\pm 2$  cc.). Return the specimen to the collecting bottle; stopper, and mix well by repeated inversion.

4. Transfer 10 cc. to a graduated centrifuge tube with a special narrow tip and centrifuge 5 minutes at 1800 r.p.m.

5. Decant the supernatant urine and remove the remainder with a capillary pipet.

6. Thoroughly mix the sediment by repeatedly drawing it up and blowing out in the capillary pipet.

7. Place 1 drop of mixed sediment on each cell of a blood counting chamber and count the casts and other formed elements, using a high dry lens. In normal urines where casts are few, 10 such drops are counted. Where the concentration of formed elements is heavy, the sediment is diluted from 1 to 5 cc. and 2 drops are counted.

8. Since, in the usual form of blood counting chamber, the ruled area is composed of 9 large squares, each of which is 1 sq. mm. in area, and since the chamber is 0.1 mm. deep, the volume contained over the total area is 0.9 c.mm. or 0.0009 cc. If 10 areas are counted, the volume of urine represented is 0.009 cc.

Assuming that 90 casts were found in this volume and that the volume of urine (or 1 per cent sodium chloride solution) in which the sediment was mixed was 0.9 cc., then  $90 \times \frac{0.9}{0.009} = 9000 = \text{number of casts in 0.9 cc.}$

But, as the casts in 10 cc. are all (presumably) concentrated in the 0.9 cc. volume, the 9000 represents the number of casts in 10 cc. of urine.

If the total volume of urine in 12 hours was 300 cc., then the total number of casts in 12-hour urine is:

$$9000 \times \frac{300}{10} = 270,000$$

The following general formula applies to the quantitative determination of all the formed elements: Number counted times volume in cc. in which sediment was mixed, divided by volume in cc. in which count was made, multiplied by volume in cc. per 12 hours, divided by 10, equals number in 12-hour urine.

9. By this method the normal urine of adults may show 0 to 5000 (average 1000) hyaline casts in the 12-hour concentrated night urine; epithelial casts are only found very rarely. Erythrocytes vary from 0 to 425,000 (average 65,760) and leukocytes and epithelial cells counted together from 32,400 to about 1,000,000 (average 322,550). According to Lyttle<sup>a</sup> the urine of normal children may show slightly more casts but slightly fewer erythrocytes and leukocytes than the average excreted in 12 hours by adults.

**Gibson's Modification.**—1. Allow no fluids on day before test.

2. Empty bladder before retiring, discard urine and note time.

3. Collect all urine passed during the night and also immediately upon awakening. Note time and measure volume.

4. Place 1 per cent of the volume of mixed urine into two 15 cc. graduated centrifuge tubes. Centrifuge urine as in Addis method.

5. Remove the supernatant fluid and adjust the volume of sediment so that the volume is

0.5 cc. if test lasted 11 to 12 hours

0.4 cc. if test lasted 9 to 11 hours

0.33 cc. if test lasted 7 to 9 hours

6. Place a drop on a hemacytometer and count the average number in one large square (0.1 c.mm.)

7. Divide the number obtained by 2. This is the number of millions of elements excreted in 12 hours.

**QUALITATIVE ANALYSIS OF URINARY CALCULI\***

**General Considerations.**—1. Urinary calculi or concretions are solid masses formed in the urinary tract from substances normally present in urine, or which may be present in excess due to faulty metabolism (cystine), or as the result of drug therapy (sulfonamide compounds). They may occur in any part of the urinary tract (renal, vesical or ureteral) and may vary greatly in size, largely because of their location, but also because of their age. The latter condition also affects their composition as well, since the stones are usually built up of concentric layers surrounding a central nucleus and in the course of their growth may affect the reaction of the urine with consequent deposition of substances differing from that of the nucleus. Simple or primary stones are encountered much less frequently than secondary or compound stones and for this reason considerably more information is obtained if, size permitting, the calculus be transected and separate analyses made of both the nucleus and at least one outer layer.

2. In the outline given below a complete examination may be made on as little as 10 mg. of powder. However, since a sample may be quite small, especially if both the nucleus and strata are to be analyzed separately, a knowledge of the general appearance of the stone as related to its composition often furnishes a clue to the selection of the proper tests to be employed, thus conserving material. Observations of the effect of heat on the sample also furnish information of value in the selection of the tests.

3. *Phosphate calculi* are nearly round, compact and the surfaces are rough. If much triple phosphate is present the texture is somewhat friable. They vary in color from chalk white to gray, occasionally showing traces of yellow. They are quite common.

4. *Carbonate calculi* exhibit nearly the same shape and coloring but are more round and harder. They occur but infrequently.

5. *Oxalate calculi* assume a large variety of shapes, often have a nodular surface and exhibit a distinct crystalline structure. They are more difficult to crush than the phosphate stones and the color may vary from grayish white to dark brown. They are much more commonly encountered than the carbonate, often in the nucleus, but not as often as the phosphate type.

6. *Uric acid and urate calculi* nearly always have a smooth rounded surface but occasionally may have a crater-like or nodular appearance. They are always colored yellow to brown, and are encountered about as often as oxalate stones.

7. *Cystine calculi* occur as oval or cylindrical concretions, having a smooth surface and a pale yellow to white color. They are rare.

8. *Xanthine calculi* are even more rare than the cystine stones. Their color varies from white to yellowish brown. They have a waxy feel.

9. Other types very rarely encountered are the light colored, fatty *urostealith calculi*, the dark colored *fibrin calculi*, the cystine-like *cholesterol calculi* and the *indigo calculi*.

**Physical Examination.**—Weigh the stone to the nearest mg. if large; to the nearest tenth of a mg. if small. Measure at least two diameters to the nearest mm.

\* See Brown, H.: *Jour. Lab. and Clin. Med.*, 24:976, 1939; Winer, J. H. and Mattice, M. R.: *Jour. Lab. and Clin. Med.*, 28:898, 1943; Kamlet, J.: *Jour. Lab. and Clin. Med.*, 23:321, 1937-38.

Describe the general shape, texture and color and after transection, the general appearance, color and prominence of the various strata.

**Preparation of Sample.**—To transect the larger stones a small bone-cutting saw is helpful. Loss of sample can be avoided if the stone be tightly wrapped in white paper before cutting. The various strata of many calculi may be readily separated from each other and from the nucleus by manipulation with forceps and pointed scissors. If the layers are poorly defined, a good sample of the outer layer may be obtained by scraping the surface with a scalpel, and a sample corresponding to the nucleus, by digging out small portions from the approximate centers of the cut surfaces. Each sample is ground in a mortar.

**Ashing.**—A small portion of powder is placed in a porcelain crucible and heated in a free flame. If there is little or no blackening and the original bulk is not appreciably altered, the calculus is mainly *inorganic*. If charring occurs, followed by nearly complete disappearance of the sample, the stone is largely *organic*. If melting and blackening occurs with formation of a tarry mass, *sulfonamides* may be expected. The color and type of flame and the odor of the burning sample are of but little help and often are confusing, although a *cystine* stone emits a characteristic penetrating odor somewhat different from that of the "burnt feather" odor of *fibrin*.

The above preliminary tests, plus the physical examination, will furnish clues to the selection of the proper confirmatory tests or they may be entirely dispensed with and the powder treated as follows:

**Chemical Tests.**—*Solution A.*—To about 10 mg. of powder in a 100 cc. Erlenmeyer flask add about 50 cc. of 5 per cent hydrochloric acid. Observe for effervescence. Heat on a boiling water bath for 10 to 15 minutes and filter while hot. The filtrate is evenly distributed among 8 test tubes. Add to each tube a small bit of litmus paper.

*Solution B.*—About 10 mg. of powder are shaken with 10 cc. of chloroform. Allow the stoppered flask to stand at room temperature until the insoluble material has settled out. Decant the chloroform solution. Save both the solution and the residue.

**Carbonate.**—If effervescence occurred on the addition of the hydrochloric acid solution the reaction is positive.

**Ammonium.**—To tube No. 1 of solution A add 10 per cent sodium hydroxide solution drop by drop, with sidewise shaking, until the litmus paper just turns blue. Add 1 cc. of Nessler's reagent. A deep yellow to brown coloration or precipitate is a positive reaction.

**Uric Acid.**—Neutralize tube No. 2 as above. Add an equal amount of 2 per cent sodium cyanide solution. Add 0.5 cc. of Folin's uric acid reagent. A blue color appearing within 5 minutes is a positive reaction. The formation of a pale blue color on long standing is to be disregarded.

**Calcium Oxalate.**—Neutralize tube No. 3 as above. Add 10 per cent acetic acid until the litmus paper turns red. A permanent white precipitate that will not redissolve on thorough shaking is a positive reaction.

**Calcium.**—Neutralize tube No. 4 as above. Acidify with acetic acid. Add 2 cc. of 4 per cent ammonium oxalate. A dense white precipitate, heavier than that remaining in tube No. 3, is a positive reaction. Or, if no precipitate remains in tube No. 3, the ammonium oxalate may be added directly to that tube.

**Phosphate.**—Neutralize tube No. 5 as in No. 1. Add 1 cc. of molybdate solution No. 1 (see page 52). Shake and add 0.5 cc. of 0.25 per cent aminonaphthosulfonic



acid solution. A blue coloration developing within 5 minutes is a positive reaction.

*Sulfonamide*.—Neutralize tube No. 6 as above. Add 1 cc. of freshly prepared 0.5 per cent sodium nitrite solution. Shake and let stand 3 minutes. Add 5 cc. of a 0.25 per cent alcoholic solution of dimethyl-a-naphthylamine (Eastman). A reddish coloration is a positive reaction.

*Magnesium*.—Neutralize tube No. 7 with ammonia. Add 1 cc. of a 0.5 per cent alcoholic solution of para-nitro-benzeneazoresorcinol (Eastman). A blue coloration is a positive reaction.

*Cystine*.—To tube No. 8 add a pinch of sodium sulfite. Dissolve by sidewise shaking and add 1 cc. of uric acid reagent (see page 52). Add solid sodium carbonate, a small portion at a time, until no more effervescence occurs. At this point the litmus paper should be blue. An immediate deep blue coloration is a positive reaction. A slow formation of a pale bluish shade is to be disregarded.

*Indigo*.—A blue color imparted to the chloroform of solution B is a positive reaction.

*Urostealith*.—A few drops of solution B are allowed to evaporate on a glass slide and the residue stained with Sudan III. Fat globules noted in the low power of the microscope are a positive reaction.

*Cholesterol*.—To about 5 cc. of solution B are added 5 cc. of acetic anhydride and 0.5 cc. of concentrated sulfuric acid. A green coloration is a positive reaction.

*Cystine*.—To about one-third of the residue from solution B in a test tube are added about 2 cc. of ammonium hydroxide. Shake vigorously for a few minutes. Allow the insoluble material to settle. Evaporate at room temperature a few drops of the clear solution on a glass slide. Six-sided platelets can be seen in the microscope if the reaction is positive.

*Xanthine*.—To one-third of the residue from solution B add a few drops of concentrated nitric acid. Evaporate carefully over a free flame to dryness. Cool and moisten with a few drops of 10 per cent sodium hydroxide solution. An orange coloration, turning deep red on warming, is a positive reaction.

*Fibrin*.—To the remainder of the residue from solution B are added 2 cc. of Millon's reagent. Heat. A red precipitate is a positive reaction.

*Notes*.—1. Prolonged heating in preparing solution A is to be avoided as a slow destruction of oxalate occurs when the solution is heated.

2. Positive reactions for magnesium, ammonium and phosphorus indicate the presence of "triple phosphate" (magnesium-ammonium-phosphate) in the calculus.

3. Positive reactions for carbonate and calcium indicate calcium carbonate. If the carbonate test is negative but phosphorus is present, the calculus contains calcium phosphate.

4. A negative test for calcium with a positive phosphorous test indicates alkaline phosphate.

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## METHODS FOR CONDUCTING KIDNEY FUNCTION TESTS

**Principles.**—1. For clinical purposes the primary object of renal function tests is the detection of the presence and degree of renal insufficiency or the reduced capacity of the kidneys to carry out their functions. An ideal test would be one determining not only the degree of insufficiency but the exact mechanism involved. But the tests commonly employed are a measure of total functional capacity rather than of glomerular filtration and tubular reabsorption separately. Indeed, it appears that only the inulin clearance test is a measure of glomerular function alone as it does not appear to be either excreted or reabsorbed by the tubules and none of the tests employed measure the functional capacity of the tubules alone. While it was formerly thought essential to study renal function from the standpoint of the individual functions of the kidneys on the basis of selective injury in the various types of nephritis, this is no longer tenable since modern studies have clearly demonstrated that variations in the clinical manifestations of renal insufficiency are dependent largely upon not only renal, but upon extrarenal factors as well, such as variations in the acid-base balance and in the crystalloid and colloid osmotic balance of the blood and tissues.

2. Furthermore, it is evident that renal function tests estimate the functional capacity of the kidneys only at the particular time they are conducted and that evidences of dysfunction may be only temporary as in acute nephritis, acute infections, urinary tract obstruction, cardiac failure, etc. As a general rule glomerular function is the first to be involved. In chronic disease of the kidneys, however, such tests possess far more prognostic value and a measure of the effectiveness of therapeutic measures, since progressive evidences of dysfunction usually indicate the relentless progress of the disease.

**Methods for Conducting Kidney Function Tests.**—1. No less than fifty renal function tests have been proposed but only a few have been widely accepted. In general terms these may be classified as follows:

A. Tests based upon the retention of urea nitrogen, total nonprotein nitrogen, creatinine, uric acid and sodium chloride in the blood.

B. Tests based upon the elimination or clearance of urea nitrogen, creatinine and inulin.

C. Tests based upon the concentration (specific gravity) and dilution of urine.

D. Tests based upon the elimination of phenolsulfonephthalein.

2. Apparently the urea clearance test is one of the best for measuring the capacity of the kidneys for eliminating their excretory products, while a combined test for the volume of urine and its specific gravity is probably best for determining the capacity of the kidneys to dilute or concentrate urine.

3. Insofar as a single test is concerned it appears that urea clearance is the most satisfactory method for estimating the total active renal tissue. But in view of the multiple functions of the kidneys, it is apparent that two or more tests should be routinely employed whenever possible. Furthermore, there is a growing conviction that all one-sided tests, *i.e.*, all methods in which the blood alone or the urine is examined, depend too much on extrarenal influences such as fluid intake, diurnal diuresis, the administration of caffeine and other stimulants, severe acidosis and alkalosis, edema and large exudates "locking" urea, congestive heart failure, severe anemia, urinary tract obstruction, acute infections, hepatic disease with jaundice, etc.

4. As far as tests based upon the retention of urea nitrogen, total nonprotein nitrogen and creatinine are concerned, it is to be remembered that they are of limited value in the detection of slight degrees of renal insufficiency and particularly because of the possible wide range due to extrarenal factors as well as the practical difficulties of controlling diet, metabolic activities and the remarkable reserve powers of the kidneys. They are, however, far more valuable and reliable in the latter stages of nephritis as well as for differentiation between the effects of congestive heart failure on kidney function and those of nephritis. In this connection it appears that a retention of the inorganic sulfates, with a consequent increase of them in the blood, may occur before other tests reveal any change in kidney function. The normal range of these inorganic sulfates is from 2.5 to 5.0 mg. per 100 cc. of serum corresponding to 0.8 to 1.7 mg. in terms of sulfur.

### VAN SLYKE'S UREA CLEARANCE TEST

The blood urea clearance is an expression of the number of cubic centimeters of blood that would contain, at the time of study, the quantity of urea removed from the blood by the kidneys in one minute and excreted in the urine during that time. This is usually expressed in terms of percentage of the average normal, which varies with the body surface area and with the minute volume of urine. The technic is described on page 52.

### CONCENTRATION TESTS

**Mosenthal's Concentration (Volume and Specific Gravity) Test.**—This test is based upon the volume and specific gravity of the urine during the day and night with an ordinary food and fluid intake. If albumin is present, correct the specific gravity of each specimen by subtracting 0.003 for each gram per 100 cc.

1. The patient is permitted the usual food and fluid intake.
2. Carefully collect in one container all urine voided from 8 A.M. to 8 P.M. This should be carefully measured and the specific gravity measured.
3. Carefully collect in one container all urine voided after 8 P.M. to 8 A.M. of the following day. Carefully measure and take the specific gravity.
4. Normal values are as follows: about 1100 cc. from 8 A.M. to 8 P.M. with a specific gravity of 1.010 to 1.020; less than 725 cc. (usually 300 to 500 cc.) from 8 P.M. to 8 A.M. with a specific gravity of 1.026 or higher.

**Mosenthal's Test-Meal Method.**—This test is based upon the characteristics of the urine—chiefly the volume of the night urine and variations in specific gravity of two-hour specimens taken during the day—when the patient is on a prescribed diet. When the kidneys are normal, the volume and specific gravity of the urine vary greatly to maintain a normal concentration of body fluids in spite of the periodic intake of food and water.

1. Upon the day of the test, and preferably also the day preceding, the patient is placed upon a full diet. But at least a pint of fluid—tea, coffee or water—must be taken at each meal with no food or liquid of any sort between meals or until after 8 A.M. the following day.
2. Have the patient empty the bladder before breakfast.

3. Collect specimens of urine in separate containers every 2 hours from 10 A.M. to 6 P.M., making 6 in all.

4. Collect all urine in one container between 8 P.M. and 8 A.M. of the following day.

5. Carefully measure and take the specific gravity of all 7 specimens. If albumin is present, subtract 0.003 from the specific gravity for each gram per 100 cc. Make sure that all specimens are of the same temperature as otherwise misleading specific gravity readings may occur.

6. *Normally* the results are as follows: (a) The night urine will be much less than the total day urine. It is usually 250 to 350 cc., and will seldom exceed 400 to 500 cc.; 750 cc. is the maximum. Its specific gravity will usually be 1.018 or above. (b) The highest specific gravity recorded for the two-hour day specimens will exceed 1.018 while the difference between the highest and lowest will be not less than 8 or 9 points. If, for example, the most concentrated specimen has a specific gravity of 1.020, the most dilute will be 1.011 or less.

7. A volume of night urine exceeding 750 cc. is usually one of the earliest evidences of impaired kidney function.

8. Fixation of the specific gravity, that is, lessened variation in the specific gravities of the 2-hour specimens, is also an important sign of renal functional impairment. In advanced cases the difference between the highest and lowest specific gravities may be only 1 or 2 points. As a rule, the level at which the specific gravity is fixed approaches 1.011 as the functional impairment increases and the kidneys lose their ability to concentrate.

**Volhard and Fahr's Concentration Test.**—1. Allow no foods from the evening before the test until it is finished, with no food between meals.

2. Breakfast at 8 A.M.: Dry cereal with sugar, syrup, or honey; no milk; one egg; toast or plain bread with butter.

3. Dinner at noon: Roast beef, steak, or chops; potatoes boiled or baked; bread and butter; jam.

4. Supper at 5 P.M.: Two eggs; bread and butter; jam.

5. At 8 A.M. of the same day have the patient void and save the specimen. Thereafter have the patient void at 11 A.M., 2 P.M., 5 P.M., and 8 P.M. Save each specimen in a separate container. After 8 P.M. to 8 A.M. of the following day collect all urine in one container.

6. Note the quantity and specific gravity of each of the 6 specimens of urine.

7. *Normally*, the specific gravity of at least one specimen should be 1.030, or at least 1.025.

**Fishberg's Concentration (Specific Gravity) Test.**—This test is based upon the specific gravity of 3 specimens of urine voided at hourly intervals in the morning and after supper at 6 P.M. (considerable protein) with minimal amounts of fluid.

1. Have the patient eat a regular evening meal at 6 P.M. with a minimal amount of fluid and a considerable amount of protein.

2. Discard all urine voided during the night.

3. Save the first sample of urine voided in the morning.

4. Have the patient rest in bed for an hour when a second sample of urine should be voided and saved.

5. If possible, the patient should arise and after one hour's activity a third sample of urine should be collected and saved.

6. Accurately determine the specific gravity of each of the 3 specimens. If much albumin is present the specific gravity should be corrected by subtracting 0.003 for each gram per 100 cc.

7. Normally the specific gravity of at least 1 specimen should be 1.025 or higher.

**Fishberg's Water Function Test.**—This is a test of the water excretory function of the kidneys and is primarily a measure of the blood supply to these organs. It makes use of the normal diurnal diuresis which usually and normally begins in the early morning.

1. Omit breakfast. For dinner and supper give the usual routine nephritic diet or the diet to which the patient has been accustomed. Permit 1 glass of water after supper.

2. At 8 A.M. have the patient empty the bladder and give 1500 cc. of water. Discard the urine.

3. Collect urine in separate containers at hourly intervals, beginning at 9 A.M. and ending at 12 noon, making 4 specimens in all.

4. Carefully measure and take the specific gravity of each specimen.

5. Normally the first hour specimen is about 400 cc. with a specific gravity of 1.001 to 1.003. Thereafter the volume is less with higher specific gravity amounting to about 100 cc. with a specific gravity of 1.012 to 1.016 at the fourth hour. The total volume of urine should be 80 to 120 per cent of the intake of water (about 1200 cc.).

**Lashmet and Newburgh's Concentration Test.**—This test is based upon the specific gravity of urine voided during the day with the patient on a special diet with no fluids.

1. After 10 P.M. of the day preceding the test, all fluid and food except a special diet are withheld from the patient. This special diet contains protein 40 gm., fat 104 gm., carbohydrate 240 gm., and sodium chloride 1 gm. It is equivalent to 1900 calories.

2. At 8 A.M. of the test day, during which only the above diet is administered, empty bladder and discard urine.

3. Collect all urine from 8 A.M. to 8 P.M. as specimen 1.

4. At 10 A.M. the following day collect urine as specimen 2.

5. At 12 noon collect urine as specimen 3.

6. Determine specific gravity of the 3 specimens.

7. Under the conditions of this test normal kidneys are able to concentrate the urine to a specific gravity between 1.029 and 1.032, while diseased kidneys cannot. The lower the concentrating ability, the greater the renal damage.

**Addis and Shevky's Concentration Test.**—This test is based upon the specific gravity of urine passed from 8 P.M. to 8 A.M. after the patient abstains from fluids for approximately 24 hours.

1. The patient abstains from fluid approximately 24 hours (after breakfast of one day until arising the next day).

2. Urine voided during the first 12 hours is discarded.

3. Urine voided during the last 12 hours (8 P.M. to 8 A.M.) is collected and the specific gravity determined.

4. Normal kidneys show a specific gravity above 1.026 (100 per cent) and 1.028 or above in 95 per cent. The average is about 1.032.

## PHENOLSULFONEPHTHALEIN TESTS

**Principles.**—The elimination of phenolsulfonephthalein is normally independent of urine volume. About 40 per cent of the dye is retained in the liver and the balance eliminated by glomerular filtration and tubular excretion. These tests should not be conducted if the patient is taking saline cathartics as they tend to delay elimination of the dye. The tests may be used for testing each kidney separately. Ureteral catheterization, however, may cause some inhibition of excretion.

**Rowntree and Geraghty's Intramuscular Test.**—1. Give the patient 300 to 400 cc. (about 2 glasses) of water to promote diuresis. Smoking and the drinking of coffee or tea should be forbidden at least 2 hours prior to and during the test.

2. Twenty minutes later have the patient empty the bladder and discard the urine. In case of necessity, catheterize.

3. Then inject *intramuscularly* exactly 1 cc. (6 mg.) of the sterile phenolsulfonephthalein solution.

4. Exactly 1 hour and 10 minutes later have the patient empty the bladder (or catheterize) and save the urine.

5. Exactly 1 hour later (2 hours and 10 minutes after the injection) have the patient empty the bladder (or catheterize) and save the urine.

6. In a graduate or volumetric flask, dilute each of the specimens of urine with water to about 800 cc. Add about 5 cc. of 10 per cent sodium hydroxide solution or enough to bring out the maximum purplish-red color; add water to each to 1000 cc. and mix thoroughly.

7. Prepare a standard by diluting exactly 1 cc. of 0.6 per cent phenolsulfonephthalein solution with about 800 cc. of water. Alkalinize with sodium hydroxide solution to obtain the maximum color and dilute to 1000 cc. with water.

8. Filter the diluted and alkalinized specimens of urine and compare with the standard in a colorimeter. The Dunning colorimeter (Fig. 95) consists of 13 sealed ampules containing standard color solutions of different percentages, an open ampule in which the unknown specimen is placed, and a small box in which the specimen is compared with the standards. It is very satisfactory for office work because the physician need not make his own standard solution. The colors remain satisfactory for over a year with very little fading when kept in the dark.

9. Otherwise, the Duboscq colorimeter may be used with U set at 10 mm. If V equals the volume of urine in cc. to which it was diluted and S the standard, the percentage of dye excreted equals  $\frac{SV}{100}$ .

10. Normally the kidneys eliminate 40 to 60 per cent of the dye in the first hour urine and 20 to 25 per cent in the second hour specimen with a total elimination of 60 to 85 per cent.

11. If the urine is collected separately from each kidney by ureteral catheterization, the dye first appears normally in 5 to 10 minutes after its intramuscular injection.

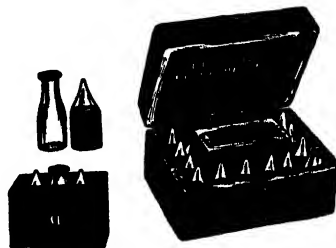


FIG. 95.—DUNNING COLORIMETER

**Shaw's Intravenous Test.**—This test is, especially useful when the patient has general edema. The total elimination may be normal in cases of acute nephritis, but an elimination of less than 25 per cent of the dye in the first 15 minutes after its injection may be the earliest evidence of renal impairment.

1. The patient empties the bladder and drinks 600 cc. of water. The urine is discarded.

2. Within an hour inject 1 cc. (6 mg.) of sterile phenolsulfonephthalein solution *intravenously*.

3. Have the patient void urine exactly 15, 30 and 60 minutes after the dye is injected.

4. Prepare a standard by diluting exactly 1 cc. of 0.6 per cent phenolsulfonephthalein solution to 1000 cc. with distilled water made alkaline by the addition of sodium hydroxide solution.

5. Determine the dye content with both specimens of urine as follows: Add to each specimen a 10 per cent solution of sodium hydroxide slowly, stirring until the maximal red color is obtained. Dilute the alkaline urine to a volume between 100 and 1000 cc. so that the color of the standard is approximated. Determine the percentage of elimination by means of the Duboscq or Dunning colorimeters as previously described.

6. Under normal conditions the dye first appears in the urine in 3 to 5 minutes. The total output for each kidney is more important than the time of appearance. This, for the 2 kidneys together, is about 35 to 45 per cent in 15 minutes; 50 to 60 per cent in the first half hour; and 65 to 80 per cent in the first hour. When one kidney is defective the time of appearance of the dye is delayed and the total elimination is reduced. Under such circumstances the other kidney may compensate to a greater or less degree by increased elimination.

The test may be applied to the two kidneys separately as follows: 1. Give the patient 2 glasses of water about  $\frac{1}{2}$  hour beforehand.

2. Introduce catheters into the 2 ureters.

3. Immediately inject the phenolsulfonephthalein solution intravenously.

4. Collect urine directly into 2 test tubes which contain a few drops of 10 per cent sodium hydroxide solution 15, 30 and 60 minutes after injection.

5. The time of first appearance of the dye in the urine, indicated by the appearance of a red color in the tubes, is noted. When the catheters are removed care should be taken to ascertain whether any urine has leaked past them into the bladder as this accident would confuse results.

6. The percentage of dye in each specimen is determined as previously described. Normally the dye first appears in 3 to 5 minutes, although it may sometimes be delayed for one or both kidneys as a result of reflex inhibition due to ureteral catheterization. The total output for each kidney is more important than the time of appearance.

### CONGO RED TEST FOR AMYLOIDOSIS AND NEPHROSIS

**Principles.**—Since Bennhold discovered that Congo red injected intravenously is rapidly absorbed by amyloid deposits in advanced amyloidosis, during the past several years the test has been used very extensively for diagnostic purposes, especially for the detection of amyloidosis in patients with chronic suppurative diseases. Further:

more, in lipoid nephrosis it is more rapidly excreted into the urine than in normal individuals.

**Procedure.**—1. Inject intravenously 1 cc. of a 1 per cent aqueous solution of Congo red per 10 pounds of body weight.

2. Exactly 4 minutes later, and again at the expiration of 1 hour, remove 10 cc. amounts of blood and place them in clean, dry test tubes for coagulation and the separation of serum which should be centrifuged. Or, each specimen of blood may be collected in test tubes carrying 20 mg. of powdered potassium oxalate and thoroughly mixed for the separation of plasma.

3. Urine should be collected at the end of 1 hour. Note the color.

4. Centrifuge the bloods to obtain clear serum or plasma.

5. Compare the 2 specimens in a colorimeter, using the 4-minute sample as the standard. Set the standard at 10 mm. and note the reading of the 1-hour sample.

6. Calculate as follows:

$$\frac{\text{mm. 1-hour sample}}{\text{mm. 4-minute sample}} \times 100 = \text{per cent dye remaining in the plasma}$$
$$100 - \text{per cent in plasma} = \text{per cent of dye disappeared.}$$

7. In normal individuals less than 40 per cent of the dye disappears from the blood in 1 hour. In amyloidosis the disappearance of 60 to 100 per cent of the dye in 4 minutes is a consistent finding. In some cases of amyloidosis, however, it is necessary to examine blood 2 minutes after the injection of the dye and compare the results with the 4-minute specimen. Since small amounts of amyloid may not absorb more than 40 per cent of the dye in an hour, the absorption of 40 or less per cent does not exclude amyloidosis.

8. In lipoid nephrosis, from 40 to 60 per cent of the dye disappears from the blood in an hour. If the urine shows the presence of the dye with the disappearance of more than 40 per cent in the blood, nephrosis is the most probable diagnosis.



## METHODS FOR THE EXAMINATION OF SALIVA

**Principles.**—1. From the clinical standpoint examinations of specimens of saliva are of most interest in relation to alterations in the amounts secreted and their chemical composition. Bacteriological and mycological examinations also possess clinical value.

2. Saliva is secreted mainly by the parotid, submaxillary and sublingual glands with small amounts contributed by small glands scattered over the buccal mucous membrane. The cells of the parotid gland are of the serous variety with the secretion of a thin watery fluid, while those of the submaxillary and sublingual glands are a mixture of serous and mucous types with the secretion of a slightly viscid watery fluid. Secretion is mainly excited through the mediation of nerves, probably through the agency of a substance liberated at their endings in the glands. This serves for the immediate need of the secretions for the mastication and digestion of foods. Otherwise, secretion is controlled by hormones. The salivary cells, however, are by no means unsusceptible to chemical influences, for a number of substances, *e.g.*, drugs and abnormal metabolic products, reaching them through the blood are capable of influencing their activity.

### COLLECTION

1. Clean the teeth, gargle with a mild antiseptic and rinse thoroughly with water. Chew a piece of paraffin of comfortable size and collect the saliva in two successive portions of 6 to 8 cc. each. Do not collect nasal mucus. The first sample contains most of the food particles and débris and should be discarded. The second sample is used for examination.

2. Or, if the reaction is not to be determined, the mouth may be prepared as described and the tongue touched with a small glass rod dipped in 5 per cent acetic acid. Withdraw saliva from under the tongue by means of a pipet or medicine dropper.

### PHYSICAL EXAMINATIONS

**Amount and Reaction.**—Normally from 1200 to 1500 cc. of mixed saliva is excreted in 24 hours. When collected with precautions against the loss of CO<sub>2</sub>, the reaction is slightly acid with a pH varying from 5.75 to 7.05 and in relation to the CO<sub>2</sub> content of the blood; otherwise the pH of mixed saliva is about  $7.14 \pm 0.04$ . Consequently an increase of CO<sub>2</sub> in the blood results in an increase of the gas in the saliva with an increase of acidity.

Reduction or suppression of the salivary secretion is known as *xerostomia* or *ptyalism*. On rare occasions it is permanent or idiopathic but little is known of its cause. Temporary xerostoma, however, is not uncommon and may be due not only to emotional states or salivary calculi blocking the ducts (*sialolithiasis*), but to fluid loss in fevers, diabetes mellitus, diabetes insipidus, chronic nephritis with edema, severe diarrhea, severe vomiting, excessive perspiration, etc., as likewise after the administration of belladonna or opium.

An increase of saliva known as *salivation*, *sialorrhea* or *ptyalism* may be due to many causes. It is not uncommon in pregnancy, due to reflex origin or some metabolic product, as likewise in nausea, hysteria, migraine, facial paralysis, paralysis agitans, tic douloureux, emotional shock, etc., of nervous origin.

It may also result reflexly from stimuli arising in the esophagus, stomach or

duodenum, as in peptic ulcer, carcinoma of the stomach, the passage of a stomach tube, etc., as well as in hepatic and pancreatic disease. Under these circumstances the excessive saliva may escape notice, pass down the esophagus and collect above the cardiac sphincter, especially after meals, to be later brought up in gushes without vomiting or nausea and constituting *water-brash*.

Since the salivary glands respond to stimuli, painful or otherwise, salivation may be associated with abnormal conditions of the mouth including not only teething of infants but caries of the teeth and ill-fitting dentures, as well as in stomatitis, gingivitis, scurvy, purpura, pernicious anemia, etc. Furthermore, salivation may be produced by various drugs and poisons like mercury, bismuth, copper, lead, iodides, bromides, pilocarpine, potassium chlorate, tobacco, etc.

**Specific Gravity.**—Normally the specific gravity averages about 1.007. It may be determined by the use of a urinometer or special type of hydrometer but possesses little clinical interest or value.

### MICROSCOPICAL EXAMINATIONS

1. Make a smear on a glass slide and examine unstained for epithelial cells, leukocytes, erythrocytes, salivary corpuscles, yeasts, molds and other structures. The slide may be stained with methylene blue, dilute carbolfuchsin or the method of Gram and examined for the various cells, bacteria, fat droplets, etc.

2. Normally, columnar and squamous epithelial cells and salivary corpuscles are found (Fig. 96). Under pathological conditions erythrocytes, pus cells and other structures may be observed.



FIG. 96.—SALIVARY CORPUSCLES

(From Lyon, *An Atlas on Biliary Drainage Microscopy*.)

### CHEMICAL EXAMINATIONS

#### Hench and Aldrich's Salivary

**Urea Index Test.**—This test is based upon the principle that mercuric chloride combines with the urea, ammonia, and other nitrogenous constituents of saliva, chief of which is urea, to form a complex salt of mercury. An excess of mercuric

chloride is shown by the formation of red mercuric carbonate on addition of a drop of the mixture to a solution of sodium carbonate. In the saliva, urea is broken down by bacterial action with ammonium carbonate. Therefore, the mercuric chloride required for both of these materials is calculated as urea.

1. Prepare the reagent by dissolving 10 gms. of mercuric chloride in 200 cc. of distilled water. Prepare a second reagent by dissolving 5 gms. of anhydrous sodium carbonate in 50 cc. of warm distilled water; cool and filter.

2. By means of a pipet transfer 5 cc. of the saliva to a small flask or beaker.

3. Add 5 per cent solution of mercuric chloride from a buret or pipet a few drops at a time, with constant stirring, until a drop of the fluid, when added to a drop of saturated solution of sodium carbonate on a white porcelain plate, gives a definite reddish-brown color. The color should appear within about 3 seconds. If it develops more slowly, the end-point is near, but not yet reached, and a few additional drops of mercuric chloride must be added.

4. When the end-point is reached note the number of cc. of mercuric chloride solution which have been added, and multiply by 20 to find the number of cc. which would be required for 100 cc. of saliva. Record this as the "mercury-combining index".

5. The normal mercury-combining index is between 30 and 50 for 100 cc. of saliva. When there is retention of urea in the blood the index rises with the blood urea, although it lags a little behind.

6. The probable blood urea may be roughly calculated as follows:

$1.43 \times \text{salivary index} - 34 = \text{probable blood urea in milligrams for each 100 cc.}$

**Qualitative Test for Ptyalin (Salivary Amylase).**—This test is based upon the principle that a solution of starch paste is digested by saliva and the color of the mixture observed when added to a solution of iodine.

1. Prepare the reagent by adding a mixture of 1 gm. of soluble starch in 5 cc. of water to 95 cc. of water heated to the boiling-point. Stir constantly until the solution again reaches the boiling-point. Allow to cool. A few drops of toluol or chloroform may be added as a preservative if the reagent is to be kept more than a few days.

2. Place 5 cc. of starch solution in a test tube and add 5 drops of saliva. Place in a water bath at 37° C. for 15 minutes.

3. Test at intervals by mixing 1 drop of the mixture with 1 drop of Gram's iodine solution on a white porcelain spot-plate. Note the color.

4. Successive changes in the color from blue to purple to red, and finally to yellow, indicate the presence of ptyalin.

5. The digestion mixture may also be tested by the Benedict's qualitative reagent for the presence of reducing sugars in the same manner as in the case of urine. A positive reaction indicates the presence of ptyalin.

## METHODS FOR THE EXAMINATION OF SPUTUM

**Principles.**—1. Sputum consists of material usually brought up from the lungs, bronchi or trachea by coughing. The amount of normal mucus secreted by these organs is too small to excite expectoration. Strictly stated, sputum does not include saliva or the nasopharyngeal secretions but it is not always possible to exclude them in its collection.

2. As a general rule, sputum is largely composed of mucous or inflammatory exudates produced in the alveoli of the lungs, bronchi, or trachea, or from a combination of these sources. Extensive accumulations in the lung, however, can be expectorated only when there is a communication with a bronchus and when the material is sufficiently fluid for expectoration. The absence of sputum, therefore, does not exclude the possibility of accumulated materials in the lungs.

3. Sputum may be largely composed of a serous transudate in pulmonary edema. Or it may be derived primarily from adjacent parts as the pleural or peritoneal cavities, cysts of the liver, the esophagus, tracheobronchial glands, etc., by rupture into the trachea, bronchi or lungs. Consequently, sputum does not always indicate that its primary source is the lower respiratory tract. Blood may also enter the bronchi and be expectorated, due to passive congestion of the lungs, ulceration of the bronchi, or trachea, hemorrhagic inflammation involving the alveoli, bronchi or trachea or from sources communicating with the latter.

### COLLECTION

1. Patients should be instructed to collect only material brought up by coughing without admixture with saliva or secretions aspirated into the throat from the nasal cavity or nasopharynx. It is also advisable for them to thoroughly rinse the mouth with water before collection as contamination with food particles may prove misleading.

2. As a general rule morning sputum, or the entire amount collected over 24 hours, is advisable for examination. In some cases of chronic pulmonary tuberculosis there may be no cough at all but only small masses of sputum rising to the larynx and swallowed without patients realizing their significance. The sputum of infants and young children is usually swallowed unless special measures are taken with the latter to encourage expectoration.

3. As a receptacle for sputum a clean, wide-mouthed bottle with a tightly fitting cork stopper or a sputum cup may be employed. For cultures the bottle and stopper should be sterilized. Patients should be particularly cautioned against smearing any of their sputa upon the outside of the container since it may be a source of danger to the examiner. Disinfectants like phenol, formalin, etc., should not be added. In case of delay in the delivery of the specimens to the laboratory, sputa should be kept in a cold place. After examination the specimen should be destroyed by heat or disinfected with a chemical agent.

### PHYSICAL EXAMINATIONS

**Quantity.**—1. It is frequently desirable to obtain a general idea of the quantity expectorated over daily periods, but accurate measurement is seldom necessary.

2. The amount per 24 hours usually varies greatly. As previously stated, it may

be so slight in early tuberculosis as to be entirely overlooked. It is usually scanty in acute bronchitis, bronchial asthma, and in the early stages of pneumonia. Large amounts varying from 25 to 100 cc. or more are the rule, however, in chronic bronchitis, bronchiectasis, gangrene and abscess of the lungs, advanced pulmonary tuberculosis with cavitation, pulmonary edema and pulmonary hemorrhage. Likewise, following the rupture of an empyema, a subphrenic abscess or a liver abscess into bronchi when there may be sudden violent coughing with a gush of pus.

3. Alterations in the daily output are of some prognostic value. Thus increasing amounts in chronic bronchitis, bronchiectasis, tuberculosis and lung abscess indicate progression while a gradual decrease indicates healing. A sudden cessation, however, is usually due to bronchial plugging followed by a flare-up of fever and constitutional symptoms unless drainage is re-established.

**Consistency.**—1. Sputa are usually classified as serous, frothy, mucoid, or glairy, purulent, seropurulent, mucopurulent, or hemorrhagic. These terms are self-explanatory.

2. The rusty sputum of lobar pneumonia is usually so tenacious that the patient may have difficulty in raising and expectorating it and especially because of pleuritic pain associated with coughing. It is also highly mucoid and sticky in acute bronchitis as likewise following an asthmatic attack.

3. In pulmonary edema, however, it is characteristically serous and frothy with traces of blood. It is likewise usually thin and watery in bronchiectasis, tuberculous laryngitis and perforating abscesses (hepatic and diaphragmatic). In these conditions as well as in lung abscess and gangrene, it is apt to separate into three layers when allowed to stand in a tall vessel. Sputa derived from tuberculous and bronchiectatic cavities frequently show the presence of mucopurulent masses which flatten out into coin-like or “mummular” masses when mixed with water.

**Odor.**—Odor is due to the decomposition of sputum from prolonged retention and may be sweet, putrid, sour or cheese-like. In acute infections it is absent or very faint. But sputa derived from tuberculous and large bronchiectatic cavities sometimes possess a peculiar sweetish sickening odor, while in gangrene of the lung the sputum is usually so foul as to require the isolation of the patient. A foul odor may also result from necrosis of malignant tumors of the bronchi (especially carcinoma) but it is a late sign more often absent than present. Sputa from ruptured subphrenic and liver abscesses may have a fecal odor, usually due to the presence of *B. coli* or other intestinal bacteria.

**Color.**—1. The color of sputum is frequently of clinical significance. A great variety of colors may be seen as determined by the nature of the substances present.

2. When composed largely of mucus it may be colorless and translucent. Mucopurulent sputa are usually whitish-yellow and sometimes streaked with green, due to the presence of *B. pyocyaneus*, other chromogenic bacteria or old pus. In jaundice, caseous tuberculosis and slowly resolving pneumonia, it may assume a uniform bright green color due to biliverdin. A dark gray or blackish sputum is usually due to anthracosis or excessive smoking and especially of cigarettes. When due to the rupture of an amebic abscess of the liver into a bronchus, it has a characteristic anchovy-sauce appearance.

3. Of particular importance is the color in relation to the presence of blood (hemoptysis). Bright red blood, most commonly in streaks, is strongly suggestive of

tuberculosis. Blood from tuberculous cavities, however, is usually very dark if due to oozing with temporary retention. Blood-stained sputum is likewise common in bronchiectasis. Needless to state blood may be derived from the nasopharynx and tuberculous individuals frequently mistake this for blood-streaked sputum.

4. An orange or rusty red tenacious and scanty sputum is the rule in the early stages of pneumonia as likewise in pulmonary infarction; the color is due to decomposed hemoglobin. A similar color may be due to iron oxides. Later the sputum frequently becomes more fluid and "prune juice" in color. This type is stated to be characteristic of "drunkard's pneumonia" and of bad prognostic import because so likely to be due to an associated pulmonary edema. A brown color, due to altered hemoglobin, frequently results from oozing in chronic passive congestion of the lungs, especially in congestive heart failure.

### EXAMINATIONS OF UNSTAINED SPUTUM

**Procedures.**—1. Pour a portion of the sputum into a Petri dish or between two large panes of glass (former preferred) to give a thin layer.

2. Carefully examine against a black background with the aid of a hand lens.

3. Pick out portions for microscopic examination, cover with coverglasses and examine with low and high objectives with the light well reduced as in the examination of urinary sediment.

4. According to conditions examine for Curschmann's spirals, bronchial casts, Dittrich's plugs, pneumoliths, elastic tissue, "asbestos bodies", pigmented cells, myelin globules and Charcot-Leyden crystals.

**Curschmann's Spirals.**—These peculiar structures occur as small whitish or yellow wavy threads, frequently coiled into loosely or tightly wound little balls (Fig. 97). Their exact nature has not been definitely determined but microscopically they are usually found to be composed of twisted strands of mucus enclosing leukocytes (especially eosinophils) and sometimes Charcot-Leyden crystals. Their presence in sputum is fairly characteristic of bronchial asthma although not present in every attack. Sometimes they can be found only near the end of a paroxysm. However, they may occasionally be met with in chronic bronchitis or other catarrhal conditions, but usually with an underlying asthmatic tendency.

**Bronchial Casts.**—These are usually, but not always, composed of fibrin and grayish in color unless stained with hemoglobin when they are of a reddish or brown color. They vary greatly in size and are usually rolled into balls or tangled masses best detected by floating out sputum in water over a black background. They are usually small and without branching in lobar pneumonia during the latter stages, while of medium size in fibrinous or chronic plastic bronchitis. Large casts of the bronchi

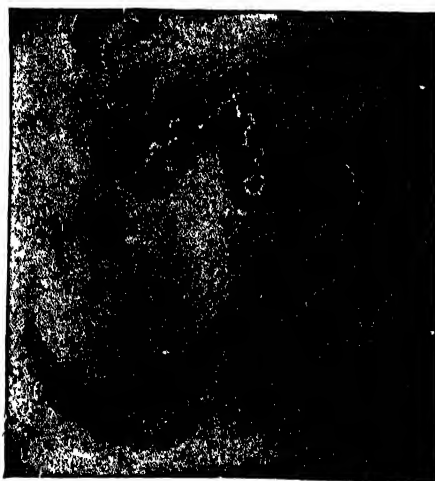


FIG. 97.—CURSCHMANN'S SPIRAL SHOWING CENTRAL THREAD (Wood)

are rare but may occur in fibrinous bronchitis and especially in diphtheria as a result of extension of the disease into the trachea and bronchi (Fig. 98).

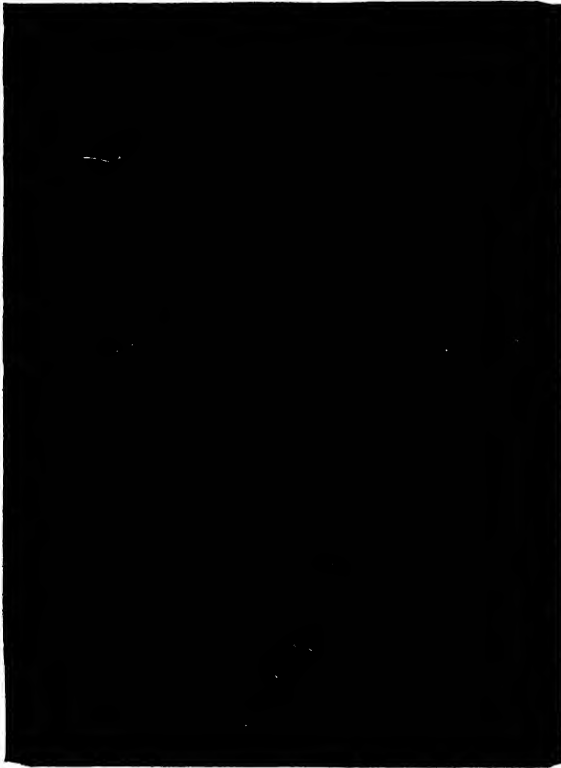


FIG. 98.—BRONCHIAL CAST (Wood)

**Dittrich's Plugs.**—These consist of yellowish or gray caseous masses, usually about the size of a pin-head but sometimes larger, emitting a foul odor when crushed. Microscopically they consist of granular debris, fat globules, fatty acid crystals and bacteria. Sometimes they are expectorated alone. They are formed in the bronchi and especially in chronic bronchitis, bronchiectasis and bronchial asthma although sometimes expectorated by apparently healthy individuals. The laity not infrequently regard them as evidence of tuberculosis. Caseous masses from the crypts of the tonsils may be mistaken for them.

**Pneumoliths.**—At times during the course of chronic tuberculosis small calcified nodules of tuberculous tissue may be expectorated and constitute the great majority of the so-called "pneu-

moliths". However, they may be composed of small foreign bodies remaining in the lung tissue or bronchi over long periods of time and encrusted with calcium salts which, upon ulceration, are expectorated, usually with hemorrhage.

**Elastic Tissue.**—Since elastic fibers are normally distributed in the walls of the alveoli and bronchioles, their presence in the sputum indicated destructive disease of these structures and consequently of very important diagnostic and prognostic significance providing they do not come from food about the teeth, which is not an infrequent source of error. They may occur in the sputum in asbestosis, abscess and gangrene of the lungs and in ulcerating malignant tumors but in the great majority of instances indicate the presence of active tuberculosis with cavitation; on rare occasions, however, they may occur in early tuberculosis before tubercle bacilli are found (Fig. 99).

**Asbestos Bodies.**—In asbestosis the sputum may show the presence of "asbestos bodies" formed in the alveoli by the deposition around asbestos fibers of an iron-containing silica gel, derived partly from the fibers and partly from the surrounding fluids. Their presence indicates exposure to asbestos dust. Elastic tissue may be present, indicative of destruction of lung tissue, with or without "asbestos bodies" or clumps.

**Pigmented Cells.**—Granules of pigment are sometimes seen in ordinary leukocytes or pus cells, but the more common and important pigment-containing cells are large mononuclear cells whose origin is still in some doubt.

The pigment is chiefly hemosiderin. To demonstrate them more clearly apply a drop of a 10 per cent solution of potassium ferrocyanide for a few minutes, followed by a drop of a normal solution of hydrochloric acid. A blue color develops (prussian blue reactin). Since such cells are most frequently found in chronic passive congestion of the lungs from congestive heart failure, they are usually designated as "*heart-failure cells*". They may also occur in pulmonary infarction, as likewise for some time after pulmonary hemorrhage; they may be so numerous as to give the sputum a brownish color. *Carbon-laden cells* are less important and characteristic of anthracosis and excessive smoking.

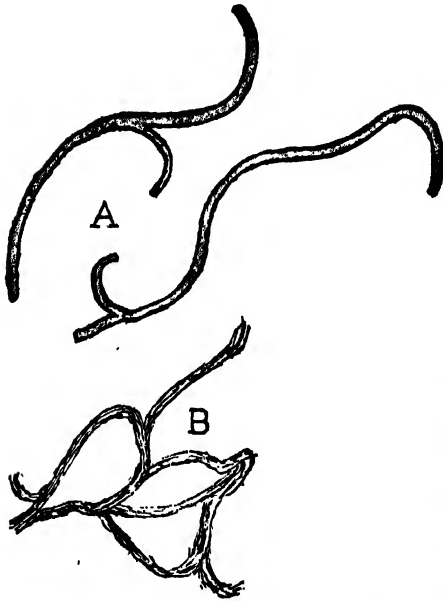


FIG. 99.—YELLOW ELASTIC TISSUE  
(Morris)

A, single fibrils, highly magnified; B, alveolar elastic tissue.

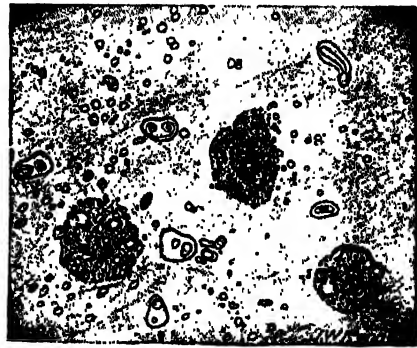


FIG. 100.—MYELIN GLOBULES. X 350

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

**Myelin Globules.**—These are colorless, round, oval, or pear-shaped globules of various sizes showing peculiar concentric or irregular spiral markings (Fig. 100). They may occur in the scanty morning sputum of apparently healthy persons, but are especially likely to occur in the mucoid sputum of bronchitis. Otherwise, however, they have little or no clinical significance except for the chances of mistaking them for more important structures, notably blastomyces.

**Charcot-Leyden and Other Crystals.**—The sputum may contain various crystals such as those of hematoidin, cholesterol and fatty acids, especially when retained with decomposition, as in bronchiectasis and abscess of the lungs. Fatty acid crystals are regularly found in Dittrich's plugs and may be mistaken not only for elastic fibers but for clumps of *Actinomyces hominis*.

The most important, however, are the colorless, pointed and often needle-like Charcot-Leyden crystals (Fig. 101) whose exact nature is still unknown. They seem to be in some way connected with the presence of eosinophils and are rarely present except in the sputum of bronchial asthma in which they frequently adhere to Cursch-

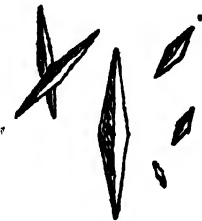


FIG. 101.—CHARCOT-LEYDEN CRYSTALS  
(Morris.)



mann's spirals. They may be absent when the sputum is expectorated but appear in large numbers after it has stood for some time.

### EXAMINATION OF STAINED SPUTUM

1. Prepare thin smears on slides and stain with Wright's stain as in differential leukocyte counts.

2. Examine for leukocytes, epithelial cells, erythrocytes, etc. Differential leukocyte counts are frequently of diagnostic aid (cyto-diagnosis).

3. The presence of an excess of *eosinophils* in the sputum of bronchial (allergic) asthma is so constant that in their absence a diagnosis of this disease is seldom justified. In "cardiac asthma" or paroxysmal nocturnal dyspnea due to left ventricular failure from hypertension, aortic stenosis or aortic insufficiency, they are almost invariably present in normal numbers. Since eosinophils are very fragile, large numbers of free granules are usually found. A simple method is to stain the dried and fixed smear 2 or 3 minutes with a saturated aqueous solution of eosin, washing with water and staining with Loeffler's methylene blue for  $\frac{1}{2}$  minute or until the thinner portions of the smear become blue. Nuclei are stained blue and eosinophilic granules a bright red.

4. *Polymorphonuclear neutrophils* are present as pus cells in every sputum, and at times the sputum may consist of little else. In old sputa the cells may be much disintegrated and difficult to recognize, when these cells predominate a pyogenic infection may be assumed.

5. *Lymphocytes* are generally present in small numbers along with neutrophils, from which they are distinguished by the possession of a single round nucleus. In early uncomplicated pulmonary tuberculosis they usually predominate. If followed by an excess of neutrophils secondary infection is strongly suggested.

6. *Endothelial leukocytes* are best studied in unstained sputum as they usually occur as pigmented cells.

7. *Epithelial cells* may come from any part of the respiratory tract and a few are always present. They occur as large squamous cells, cylindric cells (nose, trachea and bronchi) and alveolar cells.

8. *Erythrocytes* may be present in small numbers in almost any sputum. When constantly present in large numbers they are suggestive of pulmonary tuberculosis. When fresh they are usually easily recognized. They are commonly so much disintegrated as to be unrecognizable. Tests for occult blood may be required.

9. Undoubtedly the most frequent examinations of stained sputum are for tubercle bacilli. Methods, as well as bacteriological examinations for pneumococci, other bacteria, yeasts and molds, are given on pages 412 to 413 and pages 537 to 538. Various animal parasites, or their ova, may also occur in the sputum.

### CHEMICAL EXAMINATIONS

Chemical examinations are seldom conducted although the presence or absence of albumin may be of some clinical value. It is almost constantly present in the sputum in pneumonia, pulmonary edema, and tuberculosis while usually absent in bronchitis.

**Test for Albumin.**—1. Use fresh sputum, as decomposed specimens are unreliable and unsatisfactory owing to the disintegration of cells.

2. Place 10 cc. in a test tube and add 20 cc. of a 3 per cent solution of acetic acid in water.

3. Shake vigorously and filter through paper.

4. Test the filtrate by the Esbach method for the quantitative determination of albumin in the urine as described in the Section on Methods for the Examination of Urine.

5. Normally only very slight traces of albumin are present. Active cases of pulmonary tuberculosis generally show 0.2 per cent or more albumin; slightly active cases, less than 0.2 per cent. In chronic bronchitis and asthma only traces are found.

**Test for Occult Blood.**—The benzidine test described on page 155 for occult blood in urine may be employed.

## METHODS FOR THE EXAMINATION OF STOMACH CONTENTS

**Principles.**—1. Unfortunately, there are several possible sources of error capable of involving the accuracy and clinical value of stomach analyses. The passage of the tube for the removal of residuum, and again after the test meal, involves not only an important psychic stimulation to gastric secretion, but frequently entails the swallowing of considerable saliva which, because of its alkalinity, is capable of neutralizing unknown amounts of free hydrochloric acid in addition to that neutralized by the alkaline mucus secreted by the gastric mucosa which cannot be estimated by any method of analysis. Furthermore, the regurgitation of alkaline bile containing duodenal contents, which is bound to occur in a small percentage of all patients, but especially in neurotic individuals as well as those with gallbladder disease, is increased by psychic disturbances with sufficient neutralization of acid in some instances to produce a false achlorhydria. Therefore, the presence of saliva and bile in the gastric contents must be reckoned with in the clinical interpretation of acid determinations, not to mention the advisability of keeping the patient as quiet and as free as possible of anxiety and having the stomach contents removed by some one possessing adequate skill and experience.

2. Moreover, the amount of water or tea given with a test meal involves error due to dilution which, in turn, involves the emptying time of the stomach and especially when its contents are removed one or two hours after a test meal. Furthermore, the bread, soda or arrowroot crackers, or cereals, given with the meal will combine with and neutralize surprisingly large amounts of hydrochloric acid up to a certain point, following which free acid will remain in the contents, which likewise involves the important factor of emptying time of the stomach. Gross amounts of blood will also reduce acidity so that no value can be attached to free hydrochloric acid determinations in cases of actively bleeding peptic ulcers or neoplasms. And even traces of blood from trauma of the gastric mucosa, or the result of swallowing, may interfere with the clinical interpretation of occult blood tests and microscopic examinations. Occult blood may also occur in foods like the meats.

3. Under the circumstances the *kind of stomach tube* employed is a matter of importance as well as the gentleness and skill with which it is used. That of Rehfuess is known best but the Jutte tube is stated to be more readily passed with less psychic disturbance. Apparently one of the best is that devised by Miss Sawyer of the Mayo Clinic (V. Mueller and Company, Chicago) because the walls are of such thickness and size that no wire stylet is necessary as in the Jutte tube, nor is it necessary to use a metal tip as in the Rehfuess tube.

**Method for Introducing the Stomach Tube.**—1. If the patient is neurotic or has marked pharyngeal hyperesthesia, swab the fauces with a 2 per cent solution of cocaine hydrochloride.

2. The patient should be seated with the head tilted slightly forward and the clothing protected with towels or an apron.

3. Place the tip of the tube on the tongue held well out and pass it back to the throat. Then the patient is encouraged to swallow quickly upon removal of the fingers, while the tube is slowly fed into the mouth. The patient should keep the lips closed and breathe deeply.

After slight discomfort in the pharynx and passage of the tube to the level of the

cricoid cartilage, practically no discomfort is felt. If the patient has difficulty, he may be given a measured portion of the water of the test meal to swallow along with the tube, as this carries the latter to the stomach with a minimum of discomfort. The amount so given is, of course, considered in the final calculation.

It is sometimes advantageous to push the tube over to the side of the mouth, back of the teeth and passing behind the last molar tooth, just as soon as the tip has passed into the esophagus. The patient should keep the mouth closed. This causes less reflex gagging and less chance of contaminating the stomach contents with mucus from the nose and pharynx.

### METHODS OF EXAMINATION

1. Either of 2 methods may be employed: (a) The older consists in giving an Ewald breakfast and removing the stomach contents 1 hour later. (b) By the newer fractional method of Rehfuess, the tube is introduced into the fasting stomach, the residuum removed, a meal given with the tube *in situ* and fractions removed every 15 minutes for 1 or 2 hours or longer.

2. If the older method is employed, the Töpfer method of chemical analysis may be used; if the fractional method is used, each portion is examined for free and total acidity, protein and any other tests decided upon.

3. The newer fractional method is recommended because it permits a study of the gastric residuum, gives much more accurate information of the chemical and enzymic activities of the stomach and permits of an estimation of gastric emptying. Both methods require fasting for 12 hours.

4. The amount of the residuum, as well as the presence or absence of retained food from the previous regular meal, or after a special meal containing easily recognizable materials (rice pudding with raisins, jam with seeds, spinach, etc.) has been given 6 or 7 hours previously, are important in relation to the motility of the stomach, especially in atony, dilatation and pyloric obstruction.

5. The usual or *one hour test meal method* is to pass the tube for the removal of the stomach residuum. The tube is then removed and the meal given because patients cannot usually swallow it with the tube *in situ*. However, some meals or other stimulating substances, like alcohol, are readily administered by injection through the tube. If, however, the matter of possible residuum is not of clinical importance, its preliminary removal may be omitted, which is particularly advisable, whenever permissible, in nervous individuals, in order to reduce to a minimum sources of error due to the swallowing of saliva and the psychic stimulation of increased gastric secretion and regurgitation of bile. After the meal (which should be thoroughly masticated) has been given, the tube is re-introduced and the stomach contents removed 1 hour afterward, counting from the beginning, not the end of the meal. The entire collection is then sent to the laboratory for examination.

This method, however, has several shortcomings. For example, the amounts of hydrochloric acid and total acidity found are not necessarily certain indices of secretory activity; furthermore, considerable variations may occur not only in different normal individuals but even in the same individual at different times. These, as well as such changeable factors as the diluting effect of the meal, the degree of duodenal regurgitation and the rate of evacuation of the stomach contents, interfere with the clinical value of the test for diagnostic purposes.

6. Therefore, since it is now well recognized that there is great variation in the time at which the maximum of secretion of hydrochloric acid and enzymes is reached, the *fractional method* of removal has come into wide use and is generally preferred. By this method 5 to 10 cc. of stomach contents are removed by suction at intervals of 15 minutes over a period of 2 to 3 hours. Its main disadvantage is the possible discomfort of retaining the tube for this period of time with considerable gagging and the swallowing of saliva by nervous individuals. Each specimen is sent to the laboratory for examination for the amounts of free hydrochloric acid and the total acidity which should be separately reported and charted in curves.

According to Rehfuess the curves of free hydrochloric acid may occur *normally* as the isosecretory (37 per cent), the hypersecretory (33 per cent) or the hyposecretory (30 per cent). In *hyperacidity* 5 different curves may occur, namely, (a) the *laval*, in which there is a sharp rise within the first hour with remainder normal; (b) the *digestive*, which resembles the normal except that it is greatly exaggerated and prolonged; (c) the *postdigestive*, in which it increases steadily throughout the entire digestive period and is maintained in the postdigestive period; (d) the *interdigestive*, which may occur independently and (e) the *plateau* curve.

Recent investigations, however, have thrown considerable doubt upon the value of the method as originally conducted with the Ewald test meal. For example, specimens removed from different parts of the stomach at the same intervals may vary considerably in acidity and particularly in individuals with gastric disease. For this reason it has been suggested that just before each 15 minute extraction the portions be sucked into the syringe and forced back several times. Probably the best method of fractional analysis is to give a series of test meals upon successive days and to remove the entire contents of the stomach on the respective days at different periods of digestion. This is not always a practical procedure in view of the discomfort given the patient and the time involved.

### TEST MEALS

Since different foods stimulate the gastric mucosa in different degrees, certain standard "test meals" have come into general use. It is customary to give them in the morning after a period of fasting. The kind of meal to be given depends to some extent upon individual circumstances.

**Ewald Meal.**—Possibly the best known and most frequently used is that of Ewald because of its blandness, consisting of either 1 roll or 2 slices of plain or toasted wheat bread (without butter), weighing about 35 grams with 300 to 400 cc. of water, or weak tea without cream or sugar. However, because of its blandness it provides but a weak stimulus to the secretion of hydrochloric acid with the result that it yields too high a percentage of cases of false achlorhydria and especially if removed 1 hour later. Furthermore, the use of rolls or bread introduces the possibility of their containing lactic acid and sarcinae. For this reason shredded wheat biscuit, soda cracker or arrowroot cookies are preferred.

**Boas Meal.**—Otherwise, the Boas meal may be used with a preliminary stomach washing the previous evening. This meal consists of oatmeal boiled in 800 cc. of water until the volume is reduced to 400 cc. However, if retention is suspected because of atony of the stomach, dilatation or pyloric obstruction, rendering the removal and examination of the residuum particularly of interest, a special meal of easily recogniza-

ble materials should be given 6 or 7 hours before, composed of rice pudding with currants or raisins, jam with seeds, spinach, etc.

**Riegel Meal.**—If a more stimulating meal is indicated, as when hypoacidity or achylia are suspected, the Riegel meal may be given. This consists of about 200 cc. of beef broth, 150 to 200 grams of broiled beef steak (which should be thoroughly masticated) and about 100 grams of mashed potatoes.

**Heckman Meal.**—If it is desired that a meal be given through the stomach tube, in order to require its passage but once, the residuum may be removed by suction with the syringe and the Heckman meal administered, which consists of 80 cc. of freshly prepared egg albumin and 130 cc. of distilled water, to which is added 2 drops of a 2 per cent solution of methylene blue, with filtration through gauze after heating to body temperature.

Although these meals may yield satisfactory results from a physiologic standpoint, there has been a great deal of discussion by gastro-enterologists as to the clinical value of the findings. The result has been an increasing tendency to study gastric function without a meal, using either a small amount of alcohol as a direct stimulant of secretion, or a small dose of histamine injected subcutaneously for the same purpose,

**Ehrman Alcohol Meal.**—As recommended by Bloomfield and Keefer, this meal consists of 50 cc. of 7 per cent solution of ethyl alcohol. It possesses the advantages of readiness of administration through the tube which thereby is introduced but once, ease of withdrawal, and the possibility of yielding more exact quantitative results since there is no insoluble or buffer-containing residue. However, it has the disadvantage of affording but little information on the motor activity of the stomach in addition to being a somewhat unphysiological method of gastric stimulation. A modification of this alcohol meal has been proposed consisting of the addition of 1 cc. of a 0.1 per cent solution of phenolphthalein in 95 per cent alcohol to 49 cc. of 7 per cent alcohol. This is believed to make possible a determination of the acidity of pure gastric juice and of the exact emptying time, the stomach contents being removed 40 minutes after the administration of the meal and sent to the laboratory for an estimation of acid by the Dunning colorimeter. But, while it is simple and rapid to perform, toxic reactions may occur with the test contraindicated in patients with hematemesis. On the other hand, the plain alcohol meal yields the two most essential items of information, namely, the acidity of the pure juice and the volume of gastric secretion with the advantage of being administered through the tube. The same is true of the *Lewin meal* composed of a mixture of alcohol and bouillon.

**Histamine Meal.**—This method is particularly of value for differentiating between true and false achylia. It provides not only an adequate and physiological stimulus, since it may be not only a normal constituent of the gastric mucosa but likewise a gastric secretory hormone, but also the maximum of secretion (especially of hydrochloric acid) with similar results on repeated tests. And while it has been objected to on the basis that it does not stimulate the pepsinogen producing glands, yet the results observed have firmly established its many advantages, particularly since it stimulates the production of hydrochloric acid more effectively than the Ewald and alcohol meals.

1. The patient is requested to fast for at least 12 hours (over night) and to lie in bed on the back or left side.

2. The tube is passed and the gastric residuum removed. The Ewald meal may be given then or the test conducted without it.

3. A standard amount of histamine is injected subcutaneously in dose of 0.1 mg. per 10 kilograms (22 pounds) of body weight. A total dose of 0.25 mg. may be used with safety. Each 0.1 mg. of histamine is equivalent to 0.19 mg. of histamine phosphate. As a reaction of flushing, increased pulse rate, and sometimes physical discomfort, develops rapidly, caution must be exercised in the selection of patients as the test should not be used routinely.

4. Gastric secretions are removed 10 minutes later and subsequently at 10-minute intervals over as long a time as desired (usually 1 to 2 hours). All specimens are sent to the laboratory for measurement of their amounts and titration for free hydrochloric acid, total acidity, etc.

5. However, since one negative histamine test is not conclusive evidence of a true or absolute achlorhydria, a *double histamine test* has been proposed by Rivers, Osterberg and Vansant, for determining not only the maximum potentiality of acid and pepsin secretion of the gastric mucosa, but also the capacity for maintaining the increased secretory rates over relatively long periods of time. It is conducted as follows:

The gastric residuum is removed as completely as possible by several aspirations at 10- or 15-minute intervals. Histamine (0.1 mg. per 10 kg. of body weight) is then injected subcutaneously, following which the gastric juice is aspirated for 6 periods of 10 or 15 minutes each. At the conclusion of the 60 or 90 minutes, a second dose of histamine similar to the first is injected and specimens again collected at 10- to 15-minute intervals. Normally there is an initial rise in acidity, followed by a reduction about 60 to 90 minutes after the first injection; a second injection produces a second rise.

### FRACTIONAL GASTRIC ANALYSIS

**Principles.**—If properly carried out this constitutes one of the most valuable gastro-enterological examinations. Improperly and indifferently performed, it is of little value. The following classification represents a summary of the information which may be obtained by this method.

1. Measure of gastric work:

(a) Secretory function: acid and enzyme determination.

(b) Motor function: examination of fasting residuum for food and determination of the emptying time of the Ewald meal.

2. Indication of intragastric disease:

Addition to the gastric secretion of abnormal elements such as: blood (microscopic or occult), pus, mucus (of stomach origin), exfoliated epithelium, bacterial colonies, tissue fragments and foreign bodies.

3. Indications of extragastric disease:

(a) Extragastric type of acidity curve (indirect evidence).

(b) Products of extragastric disease (direct evidence):

(1) Swallowed pus, blood or mucus as indicative of lesion higher up.

(2) The presence of bile in the stomach residuum or constant regurgitation of bile during the analysis is suggestive of abnormal motor activity of the pylorus and duodenum. Pathological products in this bile residuum as blood, pus, mucus, exfoliated gall tract epithelium, cholesterol crystals, and numerous bile-stained organisms may be due to disease beyond the pylorus.

**Procedure.**—1. Patient may be instructed to take a meal 12 hours preceding the examination. A dish of rice and raisins, a meat sandwich and 30 raisins or a meal to include 4 stewed prunes will be satisfactory as the plan is to ingest some heavy cellulose which will be readily recognized in the gastric residuum the following morning upon inspection.

2. The teeth should not be brushed on the morning of the examination to exclude any possibility of swallowing blood.

3. A gastroduodenal tube is passed to a point 56 cm. from the lips as described above. Note should be made of the amount of gagging and retching which accompanies the passage of the tube. The reason for this observation is twofold. When there is considerable gagging and retching, bile is frequently regurgitated into the stomach. Recently regurgitated bile will be of lemon yellow tint. Bile, after being in the stomach, becomes of a greenish turbid hue due to the action of the hydrochloric acid. Consequently the finding of some lemon yellow bile in the stomach is usually due to regurgitation during the passage of the tube and is of no significance.

4. Extract all of the residuum with as little traumatism as possible. Measure the quantity and save for examination.

5. Give the Ewald test meal with the tube *in situ*, i.e., 35 grams of bread without the crust or a shredded wheat biscuit and 350 cc. of water. Instruct the patient to thoroughly masticate the bread or shredded wheat before swallowing.

6. Any saliva which forms in the mouth after the meal is finished is to be expectorated into a basin and must not be swallowed. The irritation of the tube in some patients will cause an almost constant flow of saliva. If it is swallowed it will greatly reduce the stomach acidity. The amount of saliva expectorated in the two-hour period is measured and recorded. Normally from 25 to 50 cc. or less will be expectorated. If 200 cc. or more are obtained, hyperptyalism is present, which may constitute evidence of vagotonia. The saliva may be examined microscopically and chemically for enzyme content to eliminate any disease of the salivary glands if there are pointings in that direction.

7. An extraction should be made every 15 minutes or the test may be simplified by making extractions every 30 minutes. An amount approximating 10 cc. should be withdrawn with each extraction in order that a sufficient quantity of juice will be available for any special examinations which may be desired. In making the extractions, as little traction on the syringe as possible is desirable. If considerable suction is made on the syringe when the tube is collapsed, the mucosa will be traumatized and occult or gross blood will be the result. If such traumatism has been caused it is well to note the fact so that the occult blood reactions may be properly interpreted. It will be remembered that one of the important points to be learned from the fractional examination is the motor power of the stomach. With this in mind there are two methods of terminating the examination:

The method recommended is to withdraw specimens every 15 minutes for a period of 2 hours from the time of ingestion of the meal. Then at the end of 2 hours the stomach is emptied by the syringe and the amount of chyme left in the stomach is measured and recorded. A food residue of much over 5 cc. at the end of the 2-hour period is indicative of delay in emptying. The amount of the residue will indicate the degree of hypomotility. After the stomach has been emptied with the syringe, a lavage of 250 cc. of water should be made. Allow that amount of water to run in and out.



This will give positive information as to the emptiness of the stomach and will act as a check on the emptying of the stomach with the syringe.

The other method consists in withdrawing 15-minute extractions until the stomach is completely empty to get the measure of motility of the stomach. This procedure takes considerably longer in the hypomotile cases and is not necessary if one gages the amount of food remaining in the stomach at the end of 2 hours and learns to compute the degree of motor impairment. The first procedure also has the advantage that it requires less time and is less tiring to the patient.

8. The following data should be recorded concerning each sample which is extracted:

(a) A record is made of the amount of secretion which is withdrawn at each extraction. It is well not to remove more than 10 cc. each time in order to allow the bulk of the food to be acted upon by the stomach. At the end of 2 hours the stomach is emptied. In order to be sure that the stomach is empty the patient should be told to assume various positions, aspiration being done in each position, *i.e.*, on the back, abdomen, right side and left side. The amount withdrawn at this last extraction is also recorded. The sum of the amounts withdrawn throughout the examination subtracted from the amount of liquid given with the meal will represent roughly the amount of fluid which has passed through the pylorus in 2 hours. This, of course, does not allow for the amount of juice secreted.

(b) A rough estimation of the amount of food withdrawn with each extraction should be made. This will give some idea of the motility of the stomach. If after an hour or an hour and one-half no food is obtained with the extraction, rapid emptying must be suspected, providing the tube is in the right position. If a residue at the end of 2 hours shows more than 10 cc. of food at the bottom of the glass, the stomach emptying power is delayed. In the presence of an abnormal amount of food at this time, it is often impossible to extract all of the stomach residue. For this reason it is well to routinely lavage the stomach with 250 cc. of water after the last extraction has been made. The amount of food residue in the return wash is recorded. Finding more than 10 cc. of food in the return wash and the last extraction combined indicates hypomotility. The more above 10 cc. the greater the degree of delayed emptying or obstruction. This is one of the most important steps in fractional gastric analysis. Too often in routine hospital and office work the examiner is satisfied with a report of the acidity alone, which constitutes only a minor part of the information which may be obtained if this test is carried out properly. This method of testing the emptying time of the stomach is just as accurate as the 6-hour barium x-ray meal and can be carried out often when an x-ray examination is not feasible. It checks with the x-ray method in the severe cases of obstruction and is a more refined and accurate method where the delay is slight.

(c) A record is made of the color of each extraction. Bile, regurgitating into the stomach, gives the stomach juices a yellow or greenish tint. The amount of bile should be recorded. It should be gaged by the intensity of the color, and recorded as plus 1, 2, 3 and 4. Bile may be found in the normal stomach.

Gross blood in the stomach often gives the contents a diffuse brown tint. A sufficient amount of blood to cause its macroscopic appearance in the stomach juice means serious disease of or about the stomach, unless it has been swallowed. Bright red blood

may be due to trauma from the tube tip. This occurs more readily in the presence of disease of the gastric mucosa.

### NORMAL STOMACH CONTENTS

*Pure human gastric juice* obtained through a fistula has been found to be a colorless fluid containing 0.40 to 0.60 per cent free hydrochloric acid, a total acidity of 0.45 to 0.60 per cent, organic solids (including mucin and the various enzymes) 0.42 to 0.46 per cent, and inorganic solids 0.13 to 0.14 per cent, with a specific gravity varying from 1.006 to 1.009. Total nitrogen varies from 0.051 to 0.075 per cent. In other words, it is composed essentially of hydrochloric acid, mucin, enzymes and inorganic salts.

**The Gastric Residuum.**—The normal gastric residuum obtained by the stomach tube during an interdigestive period usually varies in *amount* from 20 to 100 cc. with an average of 30 to 50 cc. It may be slightly yellow or green in *color* due to the regurgitation of small amounts of bile. It is fluid in consistency and contains only a small amount of ropy *mucus*, derived from the stomach or nasopharynx, but normally contains no solid food particles. The *free hydrochloric acid* expressed in terms of the number of cubic centimeters of decinormal sodium hydroxide solution required for the neutralization of 100 cc. usually varies from 0 to 30 degrees (average 18.5) or from 0 to 0.1095 per cent (average 0.0675 per cent). *Total acidity* due to hydrochloric acid, hydrochloric acid combined with protein and acid salts (phosphates and carbonates) with possible traces of organic acids, varies from 10 to 50 degrees or from 0.0365 to 0.1825 per cent (average 0.1095 per cent). *Organic acids* (lactic, butyric and other volatile fatty acids) are not normally present. The same is true of *blood* unless due to that swallowed or from trauma through collection. *Enzymes* (pepsin, rennin and lipase) are present.

**The Gastric Contents After the One Hour Ewald Test Meal.**—Stomach contents removed 1 hour after the Ewald test meal, or one of its modifications, usually varies in *amount* from 50 to 100 cc. Upon standing, an upper layer of almost clear and faintly yellow fluid (due to traces of bile) forms with a lower layer of partially digested food. A small amount of *mucus* is present easily recognized by its ropy character when the fluid is passed from one vessel into another.

The reaction is normally acid but the amounts of *free hydrochloric acid* vary considerably in relation to age and sex. Thus Vanzant and his colleagues have observed that it increases rapidly from childhood up to the age of 20 years when adult values are obtained. In men this generally varies from 45 to 66 degrees declining to 30 to 56 degrees after the age of 65 years. In women the average generally varies from 35 to 51 degrees throughout adult life so that in general terms it may be stated that the free hydrochloric acid varies from 30 to 60 degrees or from 0.1 to 0.2 per cent for adults of either sex, being slightly higher in men than women.

Naturally *total acidity* also shows similar variations due to age and sex but averages 50 to 100 degrees or 0.2 to 0.3 per cent for adults of either sex. The presence of free hydrochloric acid always presupposes a normal amount of *combined hydrochloric acid*. When, however, free acid is absent, it is important to ascertain whether any acid is secreted, and an estimation of the combined acid or acid deficit then becomes of

great value. Normally the combined hydrochloric acid varies from about 10 to 15 degrees, the quantity depending upon the amount of protein in the test meal, being somewhat higher after the Riegel meal.

In this connection it is to be remembered, therefore, that hydrochloric acid may exist in the stomach not only in a free state and in combination with the proteins of foods, but also as salts of hydrochloric acid (*total chloride*) which are neutral in reaction. For this reason various investigators have considered the curve of total chloride as being more nearly representative of the true state of secretion of hydrochloric acid. During interdigestive periods the total chloride (in terms of Cl) is about 40 per cent higher than that of the blood plasma, averaging about 500 mg. per 100 cc. of residuum. Variable amounts occur in different normal individuals but the variation is not as great as in the case of free hydrochloric acid; furthermore, it is more constant than the latter in the same individual at different times. It is slightly increased during digestion, reaching from 550 to 600 mg. per 100 cc., and may continue to rise after the acid curve has begun to fall. This is due to the continued excretion of HCl with neutralization and is particularly common in patients with excessive regurgitation from the duodenum.

The estimation of total chloride in the gastric contents is, therefore, particularly valuable in differentiation between true and false achlorhydria being low (200 to 300 mg.) in the former and usually normal in the latter.

*Lactic acid* is absent unless introduced with the food; for this reason an Ewald meal made up with arrowroot cookies or shredded wheat biscuit is preferred to bread. Although lactic acid is often present early in digestion, it disappears when free hydrochloric acid begins to appear. *Blood* is not present unless swallowed or due to trauma from the tube although the possibility of traces due to its presence in the meat of the Riegel meal must be kept in mind. *Enzymes* (pepsin and rennin) are usually present unless hydrochloric acid is absent; lipase is also normally present.

**The Gastric Contents in the Ewald Test Meal by the Fractional Method of Analysis.**—The results of analysis by this method are likewise influenced by age and sex. In the case of adults of either sex, however, both the free hydrochloric acid and total acidity normally reach their maximum in 60 to 90 minutes. As previously stated, recent investigations have thrown doubt upon its clinical value because specimens removed from different parts of the stomach at the same time may differ considerably and especially in individuals with gastric disease. For this reason it has been suggested that before the removal of each fifteen-minute specimen the contents be mixed by sucking a portion into the syringe, and forcing it back into the stomach.

**The Gastric Contents in the Histamine Test.**—Widely variable results have been observed in normal individuals. Thus, when conducted without a meal the maximum ten-minute volume of gastric juice may vary from 6 to as much 35 cc. and even reach as high as 70 cc. The free hydrochloric acid generally reaches the maximum in about 30 minutes and usually continues at this level for an hour. Age and sex, however, have considerable influence. Thus the maximum total acidity in normal adult men averages about 100 degrees at 25 years of age with a progressive decline to about 67 degrees at the age of 65 years. Women of 25 years show about 82 degrees, declining to about 67 degrees at 65 years of age.

**MACROSCOPIC EXAMINATIONS**

1. The *amount* of residuum in the fasting stomach should not exceed 100 cc. It normally varies from 30 to 50 cc. An increase above 50 cc. means either hypersecretion, hypomotility or obstruction. There should be no food in the eight-hour fasting stomach. It should be remembered that a very nervous patient may swallow very large amounts of saliva during the passage of the tube and greatly increase the amount of the residuum in that manner. However, it is not difficult to differentiate mouth mucus from stomach juice.

2. *Bile* in the fasting residuum, if found repeatedly and in large quantities, is indicative of some disease if the patient is "tube broken". The commoner conditions to be thought of in this connection are hyperchlorhydria, bile tract disease, duodenal ulcer, duodenitis and rigid pylorus from stenosis or adhesions. This should not be confused with the recently regurgitated bile due to retching in the taking of the tube. Such recently regurgitated bile is lemon yellow in color and forms a yellow foam when shaken. On the other hand, bile which has resided in the stomach for a considerable period is distinctly green and turbid. Some observers claim that bile is normally found in the gastric residuum. Large quantities of turbid green bile in a retentive stomach are often due to small bowel obstruction below the ampulla of Vater.

3. *Mucus*, if of stomach origin and present in considerable quantity, is indicative of catarrhal inflammation of the stomach. It is important, in this connection, to be able to differentiate stomach mucus from swallowed mucus. If it originates in the stomach it is flaky with particles suspended in the gastric juice. On the other hand, swallowed mucus is generally in large stringy masses and floats on the top of the gastric juice.

4. *Pus*, present in sufficient quantities to be recognized, is very rare. Such a finding would point toward acute or chronic diffuse suppuration of the stomach, abscess of the stomach or rupture of an abscess into the stomach (subphrenic, retroperitoneal, pancreatic, biliary, hepatic or splenic in origin).

5. *Odor* of the gastric residuum is not usually of much importance. In cancer and severe catarrhal gastritis, a pungent disagreeable odor may be noted. Colon bacillus infection of the stomach, of course, has a characteristic odor but it is very rare. The odor of feces is usually due to bowel obstruction or to gastrocolic fistula. A very unpleasant more or less characteristic sour odor is present in fermentation of the stomach contents.

6. *Blood*, if grossly recognized in the fasting residuum, is of the utmost importance. More than 50 per cent of all cases of cancer of the stomach will show small quantities of blood in the stomach. As a rule it is changed by the stomach juices and somewhat resembles coffee grounds. The average cancer does not bleed profusely but it bleeds constantly, hence there is considerable changed blood in the stomach at any given time. Gross blood is not as common in ulcer of the stomach. Occasionally, however, it will be present in very large quantities and is often bright red in color. Esophageal varices, sclerosed stomach vessels, erosions and severe infections also give rise to free blood in the stomach but more rarely. Duodenitis, duodenal ulcer, pancreatic or biliary tract carcinoma may cause the appearance of free blood in the stomach. Injury to the mouth or esophageal mucosa in swallowing the tube and blood from the lungs and nose should be borne in mind.

7. *Food* in the fasting stomach may be indicative of atony, dilatation, or ptosis of the stomach or, what is more likely, pyloric stenosis, pyloric or duodenal adhesions, pylorospasm, mass at or near the pylorus or pressure from without. The amount of food present and the constancy of its presence will help to decide whether one of the more serious causes is the most likely.

### TÖPFER METHOD OF CHEMICAL ANALYSIS

The method of Töpfer consists in the administration of a test meal of the Ewald type followed by its removal 1 hour later.

1. Measure and record the volume of the sample. Strain through gauze or cheesecloth and place 10 cc. of the coarsely filtered fluid in each of 3 beakers or porcelain dishes labelled No. 1, No. 2 and No. 3. If sufficient contents are not obtained, use 5 cc. and calculate accordingly. Normally 50 to 100 cc. are recovered.

2. Prepare a phenolphthalein indicator by dissolving 0.05 gm. of phenolphthalein in 100 cc. of 50 per cent ethyl alcohol.

3. Prepare Töpfer's reagent by dissolving 1 gm. of p-dimethylaminoazobenzene in 100 cc. of 95 per cent ethyl alcohol.

4. Prepare alizarin red indicator by dissolving 1 gm. of sodium alizarin monosulfonate in 100 cc. of water.

5. Prepare a 0.1 N solution of sodium hydroxide.

**Total Acidity.**—This includes free hydrochloric acid, hydrochloric acid combined loosely with protein food (combined hydrochloric acid), organic acids (chiefly lactic acid), and acid salts. It is determined as follows:

1. To the 10 cc. sample of filtered gastric contents in beaker No. 1, add 1 drop of phenolphthalein indicator.

2. Add 0.1 N sodium hydroxide solution from a buret until a faint pink is produced which persists for 2 minutes (Plate IV).

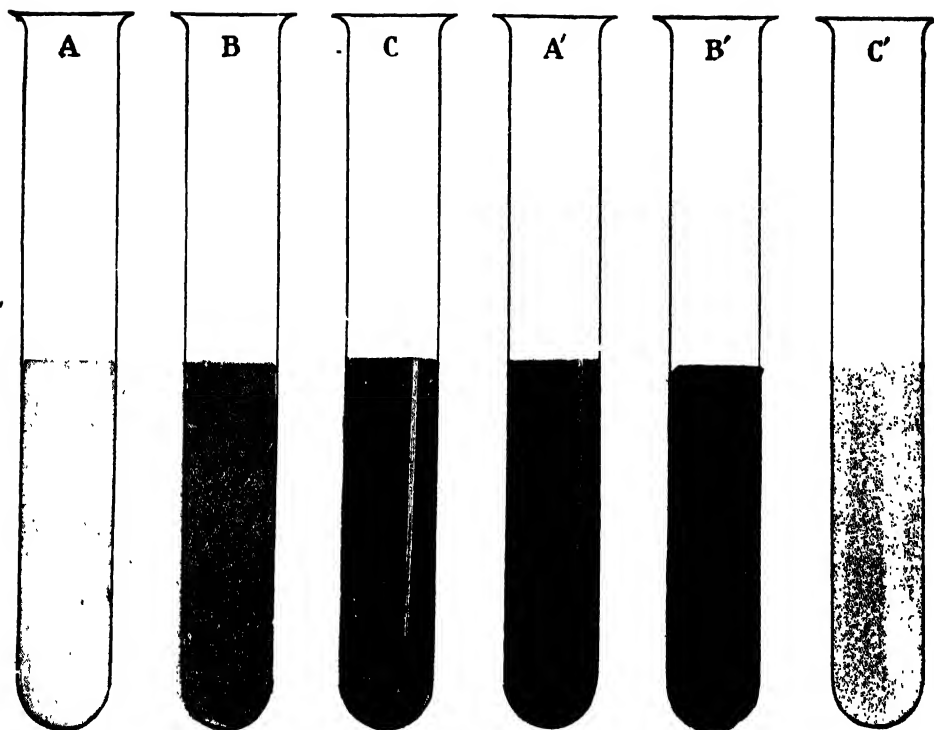
3. The number of cubic centimeters of 0.1 N sodium hydroxide solution used, multiplied by 10, gives the number of cubic centimeters of 0.1 N hydroxide necessary to neutralize 100 cc. of gastric fluid. This value is reported as expressing the total acidity. It can be converted into terms of hydrochloric acid by multiplying by 0.00365, which is the equivalent value of 1 cc. of 0.1 N sodium hydroxide in grams of hydrochloric acid.

**Free Hydrochloric Acid.**—1. To sample No. 2 add 2 to 4 drops of Töpfer's reagent and titrate with 0.1 N sodium hydroxide until the initial red color becomes salmon pink (Plate IV). If there is an initial yellow color on adding the indicator, no free acid is present.

2. The number of cubic centimeters of sodium hydroxide solution used, multiplied by 10, gives the value for 100 cc. of the gastric juice. Occasionally Töpfer's reagent gives a red color in the absence of hydrochloric acid, due to a large increase in the organic acids, especially when lactic acid is over 1 per cent and albumoses are present.

3. In case the amount of gastric juice is small, the same specimen may be used to determine the total acidity. After the end point is reached for free hydrochloric acid, add 2 drops of phenolphthalein indicator and continue the titration with 0.1 N sodium hydroxide until the persistent pink end point of total acidity is reached. The number of cubic centimeters of hydroxide used in the determination of free hydrochloric acid,

## PLATE IV



*A*, gastric fluid to which a 1 per cent solution of phenolphthalein has been added; *B*, gastric fluid to which a 1 per cent solution of alizarin has been added; *C*, gastric fluid to which a 0.5 per cent solution of dimethylamino-azobenzol has been added; *A'*, *A* after titration with a decinormal solution of sodium hydroxide; *B'*, *B* after titration with a decinormal solution of sodium hydroxide; *C'*, *C* after titration with a decinormal solution of sodium hydroxide (Boston).

(From Todd and Sanford's *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)



plus the additional cubic centimeters necessary to complete the titration with phenolphthalein, is multiplied by 10, giving the value of the total acidity.

**Free Acidity.**—This includes hydrochloric acid in the free state, organic acids and acid salts, but does not include the combined hydrochloric acid. It is determined as follows:

1. To sample No. 3 add 1 to 3 drops of sodium alizarin sulfonate indicator (Plate IV).
2. Titrate with 0.1 N sodium hydroxide solution. As the hydroxide is added, the initial tinge of yellow changes to red. The end point is indicated by a distinct violet color.
3. The number of cubic centimeters of hydroxide used, multiplied by 10, gives the free acidity value. Töpfer states that alizarin is sensitive to all acidity except combined hydrochloric acid.

**Combined Hydrochloric Acid.**—This value is obtained by subtracting the value obtained for free acidity from that of the total acidity. Cases are seen where there is no free hydrochloric acid but much combined acid, indicating that acid has been secreted but has combined with the food protein.

**Organic Acids and Acid Salts.**—This value is obtained by subtracting the value of free hydrochloric acid from that of the free acidity.

### REHFUSS METHOD OF FRACTIONAL CHEMICAL ANALYSIS

The reagents are the same as in the Töpfer method, except that 0.01 N sodium hydroxide is used instead of 0.1 N.

**Total Acidity.**—1. Place 1 cc. of the filtrate and 15 cc. of distilled water in a porcelain evaporating dish.

2. Add 1 drop of phenolphthalein indicator.

3. Titrate with 0.01 N sodium hydroxide solution until a faint pink, lasting for 2 minutes, indicates the end point.

4. The number of cubic centimeters of 0.01 N sodium hydroxide required to neutralize 1 cc. of the sample, multiplied by 10, gives the number of cubic centimeters of 0.1 N hydroxide required to neutralize 100 cc. of the gastric contents.

**Free Hydrochloric Acid.**—1. Place 1 cc. of gastric filtrate and 15 cc. of distilled water in a porcelain evaporating dish.

2. Add 1 or 2 drops of Töpfer's reagent; if, on adding the indicator, there is an initial yellow color, no free acid is present.

3. Titrate with 0.01 N sodium hydroxide solution until the initial red color becomes salmon pink (the end color is more definitely yellow than orange).

4. The number of cubic centimeters of 0.01 N sodium hydroxide required, multiplied by 10, gives the number of cubic centimeters of 0.1 N hydroxide required to neutralize the free hydrochloric acid in 100 cc. of the gastric contents.

### SAHLI METHOD FOR FREE HYDROCHLORIC ACID

This method requires more time but gives more accurate results because of a sharper end point. It is based on the liberation of iodine from the reagent employed in the presence of free hydrochloric acid. The iodine is titrated with sodium thiosulfate, using a starch indicator.



1. Prepare Sahli's reagent, which is a mixture of equal parts of a 48 per cent aqueous solution of potassium iodide and an 8 per cent aqueous solution of potassium iodate.
2. Prepare an 0.01 N solution of sodium thiosulfate.
3. Prepare a 1 per cent aqueous solution of starch.
4. Place 1 cc. of the strained sample and 10 cc. of distilled water in a porcelain evaporating dish.
5. Add 1 cc. of Sahli's reagent, mix and allow to stand for 5 minutes.
6. Titrate with 0.01 N solution of sodium thiosulfate until only a faint yellow color of the liberated iodine remains.
7. Add 0.5 cc. of the soluble starch solution. The mixture turns blue. Continue the titration until the blue disappears.
8. The total number of cubic centimeters of 0.01 N sodium thiosulfate used in the titration of 1 cc. of gastric juice is equivalent to the number of cubic centimeters of 0.01 N sodium hydroxide necessary to neutralize the free hydrochloric acid in 1 cc. of gastric contents. This value, multiplied by 10, represents the number of cubic centimeters of 0.1 N sodium hydroxide necessary to neutralize 100 cc. of stomach contents.

### QUALITATIVE TESTS FOR LACTIC ACID

Lactic acid is a product of carbohydrate fermentation by bacteria and yeasts. Normally it is not present at the height of digestion. Small amounts may be introduced with the test meal unless arrowroot cookies are used. It is most often present in stagnation of the gastric contents associated with deficient hydrochloric acid.

**Uffelmann's Test.**—1. Prepare the reagent by adding 10 per cent ferric chloride solution to a 1 per cent aqueous phenol solution until an amethyst color develops.

2. To 5 cc. of reagent, add 5 cc. of strained gastric juice. To another 5 cc. portion, add a few drops of dilute hydrochloric acid as a control.

3. Lactic acid produces a canary-yellow color. The reagent will detect 0.01 per cent of lactic acid. Hydrochloric acid discharges the amethyst color, leaving the solution colorless. If the gastric juice contains much free hydrochloric acid, the value of the test is decreased. Other organic acids give results similar to lactic acid.

**MacLean's Test.**—1. Prepare the reagent by dissolving 5 gms. of ferric chloride in a mixture of 100 cc. of saturated aqueous solution of mercuric chloride and 1.5 cc. of concentrated hydrochloric acid.

2. Place 5 cc. of water in a test tube as a control. In another tube place 5 cc. of gastric contents. To each add 5 drops of reagent.

3. A reddish color indicates the presence of lactic acid.

**Strauss' Test.**—1. Prepare a 10 per cent aqueous solution of ferric chloride.

2. Place 5 cc. of strained gastric contents in a small separatory funnel (Fig. 102). Add 20 cc. of ether and shake thoroughly. Let stand until the ether layer has separated, then run out the layer of gastric juice and all but the final 5 cc. of ether.



FIG. 102.—SEPARATORY FUNNEL

3. To this ether extract add 20 cc. of distilled water and 2 drops of the ferric chloride solution. Shake the mixture gently.

4. When lactic acid is present in a concentration of 0.05 per cent, a slight greenish color develops. If the concentration is 0.1 per cent, or higher, the color is an intense yellow, due to ferric lactate.

**Kelling's Test.**—This is a fairly satisfactory color test depending upon the formation of ferric lactate.

1. Fill a test tube with water.
2. Add 2 drops of a 10 per cent solution of ferric chloride to give a faint canary yellow. Mix well.
3. Pour  $\frac{1}{2}$  into a second tube for a control.
4. To one tube add 1 cc. of strained stomach juice.
5. If lactic acid is present a deep yellow color develops.

### TESTS FOR OCCULT BLOOD

**Benzidine Test.**—Provided the reagents are satisfactory, this test is a very sensitive one. Different lots of benzidine vary greatly in sensitivity and hydrogen peroxide solution rapidly loses its strength. For this reason it is always advisable to set up a positive control using water with an extremely minute amount of blood added, such as would adhere to the tip of an applicator. The test is based upon the principle that the peroxidase activity of the blood decomposes hydrogen peroxide with the oxidation of benzidine by liberated oxygen.

1. Prepare a saturated solution of benzidine crystals in glacial acetic acid. Keep in a brown bottle in a dark place. Or, prepare the reagent just before use by dissolving the crystals picked up on the point of a knife blade in 5 cc. of glacial acetic acid with the aid of gentle heating.

2. To 3 cc. of the reagent add 2 cc. of the gastric contents and mix thoroughly. Add 1 cc. of hydrogen peroxide solution (usually 3 per cent).

3. If blood is present a green to deep blue color, depending on the amount of blood, will form on adding the peroxide. Too much benzidine solution or too much peroxide interferes with the sensitivity and accuracy of the test.

4. A confirmatory test may be conducted as follows: (1) If fat is present, render the gastric specimen slightly alkaline with sodium carbonate or sodium hydroxide solution. (2) Extract in a separatory funnel with an equal amount of ether. (3) Discard the ether extract. (4) Make the residue acid with acetic acid and extract with ether. (5) Evaporate the ether extract to dryness, using a water bath which has been heated to boiling and the flame then extinguished. (6) Add 1 cc. of water, stir to dissolve the residue, then add a few drops of benzidine solution and a drop or two of hydrogen peroxide. (7) The development of a green to deep blue color indicates a positive reaction.

**Orthotoluidine Test.**—This test is based upon the principle that the peroxidase activity of the blood decomposes hydrogen peroxide with the oxidation of orthotoluidine by liberated oxygen.

1. Prepare the reagent by diluting 4 cc. of orthotoluidine with 96 cc. of glacial acetic acid. Mix thoroughly. The reagent is stable for 1 month.

2. In a test tube mix 1 cc. of reagent, 1 cc. of gastric contents and 1 cc. of 3 per cent U.S.P. hydrogen peroxide.

3. In the presence of blood a bluish-violet color develops (sometimes rather slowly) which persists for some time (several hours in some instances).

### TEST FOR BILE

**Principle.**—The following method is based upon the oxidation of the bilirubin with nitric acid to form biliverdin (green).

**Procedure.**—1. Place about 1 inch of powdered ammonium sulfate in a test tube and add 10 cc. of gastric juice.

2. Shake vigorously for a minute.

3. Add 3 cc. of acetone and thoroughly mix by inverting the tube six times (do not shake).

4. Allow the acetone to separate.

5. Allow a drop of nitric acid to flow down the side of the tube.

6. A green color is a positive reaction. If too much acid is used the biliverdin will be oxidized to a purple or red.

7. If the gastric juice is of a deep green color, dilute 4 or 5 drops with 10 cc. of water and proceed as above.

### TESTS FOR PEPSIN

**Principles.**—Pepsinogen is normally secreted by the stomach. It has no digestive power until transformed into pepsin by free hydrochloric acid (to a lesser extent by organic acids and the protein salt of hydrochloric acid). Its presence is detected by the digestion of egg albumin.

**Qualitative Procedure.**—1. Place 25 cc. of gastric juice in a small flask. If the specimen does not contain free hydrochloric acid, add a few drops of 10 per cent hydrochloric acid.

2. Place in the flask with the gastric juice a disk of coagulated egg albumin. Stopper and place in an incubator at 37° C.

3. If pepsin is present the disk will begin to swell in from ½ to 1 hour and dissolve in about 3 hours.

**PREPARATION OF EGG ALBUMIN DISKS.**—(a) Boil an egg very slowly until the albumin is distinctly coagulated.

(b) Cut the albumin into small cylinders about 5 millimeters in diameter.

(c) Section the cylinders into small disks about 1 millimeter thick.

(d) The disks can be preserved in glycerin until needed, but should be washed in water before using.

**Quantitative Procedure (Method of Mett Modified by Nirenstein and Schiff).**

—1. Introduce into a small Erlenmeyer flask 1 cc. of gastric juice and 15 cc. of N/20 hydrochloric acid (0.18 per cent hydrochloric acid).

2. Add two Mett tubes prepared as indicated below, stopper the flask to prevent vaporation and place in an incubator at 37° C. for 24 hours.

3. By means of a low-power microscope and a millimeter scale (graduated to half millimeters) determine accurately the length of the column of albumin digested at

each end of the tubes. It is well to run the determination in duplicate, in which case the result is the average of the eight figures obtained.

4. Ordinarily from 2 to 4 millimeters of albumin are digested by normal human gastric juice.

5. The peptic power is expressed as the square of the number of millimeters of albumin digested. This is based on the Schütz-Borissow law that the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested. Therefore a gastric juice which digests 2 millimeters of albumin contains four times as much pepsin as one which digests only 1 millimeter of albumin. For example, if the microscopic reading gives on an average 2.2 millimeters of albumin digested, the pepsin value for the diluted juice would be  $2.2 \times 2.2 = 4.84$  and for the pure undiluted juice,  $4.84 \times 16 = 77.44$ .

**PREPARATION OF METT TUBES (CHRISTIANSEN'S METHOD).**—The liquid portions of the whites of several eggs are mixed and strained through cheesecloth. The mixture should be homogeneous and free from air bubbles. It is best to allow the egg white to stand for 2 or 3 hours in a vacuum dessicator to remove air more completely. A number of thin-walled glass tubes of 1 to 2 millimeters internal diameter are thoroughly cleaned and dried and cut into lengths of about 10 inches. These are sucked full of the egg white and kept in a horizontal position. Into a large evaporating dish or basin 5 to 10 liters of water are introduced and heated to boiling. The vessel is then removed from the fire and stirred with a thermometer until the temperature sinks to exactly  $85^{\circ}$  C. The tubes filled with egg white are immediately introduced and left in the water until it has cooled. The tubes thus prepared are soft boiled, more easily digested than hard-boiled tubes, and free from air bubbles. The ends are sealed by dipping in melted paraffin or sealing wax (preferably the latter), and the tubes can be kept thus for a long time. When ready for use, mark with a file and break into pieces about 3 or 4 inches long. After cutting, the tubes should be immediately introduced into the digestion mixture or may be kept a short time under water. Tubes whose ends are not squarely broken off must be rejected.

The digestibility of different egg whites varies widely. Hence, in making up a new set of tubes, if we wish our results to be comparable these tubes must be standardized against those first prepared. This may be done by running simultaneous tests with tubes from the two series, using the same gastric juice and comparing the lengths of the column digested in each case. Christiansen's method of preparing tubes of the same digestibility is to be preferred. He proceeds as in the original preparation of the tubes except that as the water cools from  $90^{\circ}$  to  $80^{\circ}$  C. a single tube containing the new egg white is dropped in at each degree change of temperature, that is, at  $90^{\circ}$ ,  $89^{\circ}$ , etc. Pieces of each of these tubes as well as of the original standard tubes are then allowed to digest simultaneously in portions of the same gastric juice. One of these tubes should show a digestibility equal to that of the standard tubes. For example, the tube coagulated at  $88^{\circ}$  C. may show the proper digestibility. Then the new series of tubes should be made in the same manner as this one; that is, introduced at  $88^{\circ}$  C. The tubes thus prepared should be again checked up with the standard to see that no mistake has been made.

### TEST FOR TRYPTIC ACTIVITY

**Principles.**—Trypsin is not secreted by the stomach but occurs in the pancreatic juice. It may be found, however, in the stomach contents because of regurgitation of duodenal contents through the pylorus. *Since it is destroyed by the pepsin-hydrochloric acid of the stomach, the determination must be made immediately after securing gastric juice, especially in cases of high acidity.*

- Procedure (Spencer).**—1. Prepare five reagent tubes; more if desired.
2. To tubes 1 and 2 add 0.5 cc. of gastric contents (filter if cloudy).
  3. To tubes 2, 3, 4 and 5 add 0.5 cc. of distilled water.
  4. From tube 2 remove 0.5 cc. of its mixed contents and add to tube 3. Mix thoroughly and add 0.5 cc. from tube 3 to tube 4. Repeat for tube 5.
  5. This gives dilutions of gastric contents of 1, 1 : 2, 1 : 4, 1 : 8, and 1 : 16.
  6. To each tube add 1 drop of phenolphthalein solution (phenolphthalein, 1 gram; 95 per cent alcohol, 100 cc.); then add drop by drop a 2 per cent sodium carbonate solution until a light pink color is produced.
  7. To tubes 1, 2, 3 and 4 add 0.5 cc. of casein solution. Tube 5 must receive 1 cc. of casein solution, since it contains 1 cc. of the diluted gastric contents. For the casein solution, dissolve 0.4 gram of casein in 40 cc. of N/10 sodium hydroxide. Add 130 cc. of distilled water, then 30 cc. of N/10 hydrochloric acid. This leaves the solution alkaline to the extent of 10 cc. of N/10 sodium hydroxide, minus about 3 cc. neutralized by the casein.
  8. Incubate for 5 hours at 40° C.
  9. Precipitate the undigested casein by dropwise addition of a solution of the following composition: glacial acetic acid, 1 cc.; 95 per cent alcohol, 50 cc.; distilled water, 50 cc. The tubes in which digestion has been complete remain clear; others become turbid.
  10. The tryptic values are expressed in terms of dilution. Thus, complete digestion in tube 3 (a dilution of 1 : 4) shows four times the tryptic power of undiluted gastric juice which is taken as a standard as 1; therefore, its tryptic value is 4.
  11. Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

### TEST FOR RENNIN

**Principle.**—Rennin is an enzyme capable of coagulating the protein of milk. Fresh milk is used, therefore, as the reagent.

- Lee's Test.**—1. Place 5 or 10 cc. of fresh milk in a test tube.
2. Add 5 drops of gastric juice.
  3. Place in the incubator for 15 to 20 minutes.
  4. If rennin is present, coagulation will occur. In this test it is sometimes difficult to tell whether the rennin or the acid in the gastric juice caused the coagulation; however, rennin is practically always present where there is hydrochloric acid in the stomach and the test is only of value in those cases in which there is no hydrochloric acid, to determine the presence or absence of a true achylia.
- Biegel's Test.**—1. Place 5 cc. of fresh milk in a test tube.

2. Add 5 cc. of gastric juice neutralized with N/100 sodium hydroxide (phenolphthalein as indicator).
3. Place in a water bath at 40° C.
4. If rennin is present in normal amount, coagulation will occur in ten to fifteen minutes.
5. Delayed coagulation indicates a less amount.

### MICROSCOPIC EXAMINATION

As a general rule, microscopic examinations of the residuum and recovered test meals do not afford much information of clinical value. Occasionally, however, the findings are unexpected as, for example, the presence of ova of parasites or the latter themselves. Microscopic examinations are best carried out on the unfiltered residuum of the fasting stomach, or the unfiltered specimens obtained after a test meal.

**Procedure.**—1. Place a small drop of gastric residuum on a slide and cover with a coverglass.

2. Examine with low and high objectives with the light reduced.
3. Mix a drop of fluid with a drop of Sudan III on a slide and cover with coverglass. Neutral fat globules will be yellow or red.
4. Mix a drop of fluid with a drop of Lugol's solution on a slide and cover with coverglass. Starch granules will be blue or blue-black.
5. Make a thin film on a slide, fix with heat and stain with dilute carbolfuchsin; dry. Examine with oil-immersion objective for Boas-Oppler bacillus, staphylococci, yeast, sarcina and other microorganisms. Note the organisms present and whether in small or large numbers, singly, in groups or colonies (masses).
6. With the above preparations examine for the following:

(a) *Red blood corpuscles* found in small numbers cannot be considered of any pathological significance. Trauma from passing the tube and aspirating the stomach contents will frequently give rise to their appearance. The consistent finding of large numbers in the absence of considerable trauma may be indicative of ulcer, cancer or erosion.

(b) *Leukocytes* are frequently found in the gastric residuum. They are usually partially digested by the gastric juice. They may be indicative of disease from the nose or throat. If so, they are usually associated with other products of inflammation from that region which can easily be identified, *e.g.*, mucus and exfoliating epithelium. If these cells are deeply bile-stained and associated with a bilious residuum and other inflammatory elements also bile-stained (*e.g.*, columnar epithelium, mucus, bacteria, etc.) it is very suggestive of biliary tract disease. The presence of large quantities of pus cells with gastric exfoliative products, mucus and bacteria, would suggest gastritis of the infective type (Fig. 103).

(c) *Mucus* in small amount is practically always found in the gastric residuum. A differentiation between mouth and stomach mucus can often be made by the microscopic examination. Gastric mucus is usually present as small flocculations which, under the microscope, look like spherical snail-like bodies. It has a fibrillary appearance and has cellular bodies in its substance. If present in large quantities it is indicative of catarrhal gastritis. Mucus is dissolved by alkalis but not by acetic acid. Mucus from the biliary tract should be easy of identification. It occurs in wavy semi-spirals and

is rather dense in appearance and stained a deep yellow or greenish yellow if it has resided in the stomach very long. The frequent finding of bile-stained mucus in the fasting stomach is suggestive of biliary tract disease. Mucus from the nose is easily recognized by its peculiar tenacity and its staining with pigment or its grayish appearance.

(d) *Epithelium* is practically always present in the residuum (Fig. 104). The usual cell found is the squamous type. It has no special significance and is derived from the mouth, pharynx and esophagus. It is swallowed with mucus during the passage of the tube. Cellular elements from the stomach mucosa are of the columnar variety. They are rarely seen in the normal residuum. Cells are quickly digested in the stomach and unless they are present in considerable numbers they will not be found. In gastritis cellular elements from the gastric tubules are often found. The acid or parietal cells are probably the more easily recognized of the tubule cells. They are about midway in

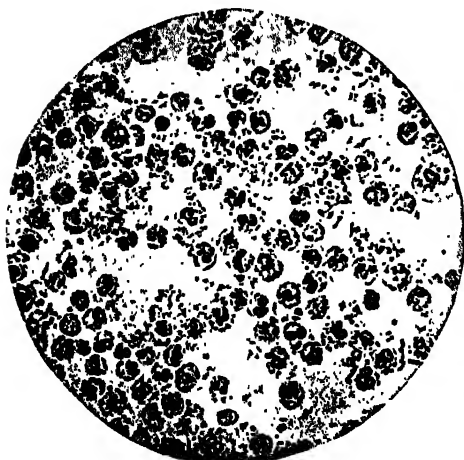


FIG. 103.—PUS CELLS IN STOMACH CONTENTS



FIG. 104.—COLUMNAR EPITHELIUM IN STOMACH CONTENTS

(From Lyon, *An Atlas on Biliary Drainage Microscopy*.)

size between a leukocyte and a squamous cell. With eosin and hematoxylin stain the acid cells are stained red and the central or peptic cells blue. The peptic cells take the stain very poorly and are very difficult to recognize, there being nothing but the nuclei remaining. They are much smaller than the acid cells, being a little larger than a leukocyte. They have a long oval nucleus often with just a shred of protoplasm attached. The acid cells, on the other hand, have a very distinct nucleus and the entire protoplasm stains with a fine stippling of the granules. In chronic catarrhal gastritis exfoliating elements from the gastric mucosa may be found, even without a severe grade of atrophy being present. The association of these cellular elements from the gastric mucosa with colonies or groups of pathogenic organisms is indicative of an infective type of gastritis. The frequent presence of deeply bile-stained columnar epithelium in the fasting stomach associated with bile is suggestive of gall tract disease.

(e) *Bacteria* in the gastric residuum are rarely of importance. In the ordinary

tube examination a few bacteria will usually be found. However, they will be associated with oral or nasal epithelium or mucus. In other words, they have been swallowed during the passage of the tube. The presence of bacteria, particularly of the pyogenic variety, occurring in colonies or masses and intimately associated with exfoliating epithelium from the stomach, usually means an infective type of gastritis. The presence of masses of deeply bile-stained organisms, on the other hand, may indicate biliary tract disease.



FIG. 105.—OPPLER-BOAS BACILLI

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

The finding of the Boas-Oppler bacillus in the stomach is significant of gastric stagnation. In a great majority of the early cases of cancer the Boas-Oppler bacilli will not be found. It is not until the hydrochloric acid starts to be reduced and obstruction has occurred that one can expect to find this organism (Fig. 105).

(f) *Tissue fragments* are occasionally found in the gastric residuum. Rarely in cases of cancer, small fragments of mucosa may be found showing carcinomatous infiltration and probably more often areas of necrotic tissue containing leukocytes and bacteria. In achylia gastrica and in atrophic gastritis fragments may be recovered showing a great diminution in the number of gastric tubules or a complete



FIG. 106.—STARCH GRANULES IN STOMACH CONTENTS



FIG. 107.—STRIATED MUSCLE IN STOMACH CONTENTS

(From Lyon, *An Atlas on Biliary Drainage Microscopy*.)

absence of tubules. A small round cell infiltration of the fragment may be noted in cases of gastritis. Small fragments of gastric mucosa may be found in peptic ulcer. This rarely happens, however, as the hyperacid juice, commonly found in this condition, soon digests any protein matter present. The finding of fragments of mucous membrane showing a hyperplasia of the glandular elements particularly of the base of



the gland would suggest a diagnosis of hypertrophic glandular gastritis. Einhorn describes the recovery of hemorrhagic flakes of gastric mucosa in the fasting residuum which he ascribes to "gastric erosions".

(g) *Starch granules* are usually present and easily recognized by their concentric striations. When undigested, they stain blue with Lugol's solution; when partially digested, a reddish color due to erythrodextrin. Fat may be present, as likewise other particles of partially digested food such as muscle fibers and vegetable cells. Various crystals may be found, especially of fats, but they possess no particular significance. Animal parasites or ova may be observed (Fig. 106).

(h) *Striated muscle fibers* (Fig. 107) and *connective tissue* are easily recognized. *Fat globules*, unstained by the iodine solution, may be stained by running water under the coverglass a drop of Sudan III; neutral fat globules stain red or yellow.

## METHODS FOR EXAMINATION OF BILE AND DUODENAL CONTENTS OBTAINED BY DUODENAL-BILIARY DRAINAGE

**Principles.**—1. From the clinical standpoint it is obvious that examinations of the bile obtained without the need of surgical intervention possess, potentially at least, most interest and value from the standpoint of diagnosis of diseases of the gall tract, although the examination of specimens obtained during operations or immediately after death are of particular value in relation to investigative work. Fortunately, it is usually possible to obtain bile preoperatively by the duodenal drainage method of Lyon.<sup>1</sup> Owing, however, to the chances of contamination with gastric and duodenal juices, the method has been severely criticized. It is true that gross contamination reduces the value of macroscopic examinations and of microscopic examinations as well, at least insofar as cytology is concerned. Contamination may likewise interfere with the accuracy of chemical analyses but nevertheless when bile is properly collected by this method it would appear that macroscopic examinations for color, viscosity, flocculi, "sand", etc., are of distinct diagnostic value as well as microscopical examinations for the kinds and numbers of cells and especially for crystals of cholesterin, calcium bilirubinate and parasites. Obviously, however, even minor contamination renders bacteriological examinations worthless, but here again it is apparently possible in many instances to obtain bile acceptable for this purpose, providing all of the precautions against contamination are taken, as so carefully described and emphasized by Lyon.

2. According to this method, the bile first obtained is from the common duct following relaxation of its sphincter and is designated as "A" bile. This is thought to be followed by bile from the gallbladder, designated as "B", and finally by that freshly excreted by the liver, designated as "C". If the bile has escaped from the common duct because of relaxation of the sphincter, its segregation into these 3 categories cannot be made and the bile collected is then assumed to be a mixture of that from the gallbladder and biliary ducts, designated as "BC". Needless to state, considerable skill and experience in the microscopy of the bile are essential in relation to the clinical value and interpretation of the results.

3. The fasting duodenal contents removed before the instillation of magnesium sulfate usually amount to about 20 cc. and are normally composed of pancreatic and intestinal secretions, a small amount of bile and some gastric contents when the latter have not been effectively excluded. From the clinical standpoint, an analysis of the duodenal contents is ordinarily limited to macroscopic and microscopic examinations, but examinations for the pancreatic enzymes (trypsin, amylase and lipase) are of great clinical value in the diagnosis of chronic pancreatitis (achylia pancreatica) and occlusion of the duct of Wirsung as well as examinations for bile in relation to cholecystitis and particularly cholelithiasis.

Examinations for mucus, pus, crystals (cholesterin and calcium bilirubinate) bacteria and parasites likewise possess diagnostic value. For example, the occurrence of considerable mucus and pus is evidence of catarrhal duodenitis or inflammation of the gall tract. Cuboidal or oval cells containing a single nucleus points to acute duodenitis or to chronic duodenitis if they are hyaline or otherwise degenerated. On the other

hand, layers of bile-stained, simple, tall, columnar epithelial cells indicate a possible cholecystitis while bile-stained, short, columnar cells point to cholangitis. *Strongyloides stercoralis*, *Giardia lamblia*, *Clonorchis sinensis* or other parasites may be found, sometimes in large numbers, as well as cystic and vegetative forms of *Endamoeba histolytica*, which indicate infestation of the liver or biliary ducts.

The presence of bile in the fasting duodenal contents, however, is clinical evidence against complete obstruction of the common bile duct. Despite repeated stimuli there may be a delay of many hours before the flow of bile is established. This may be due to dislodgment of a stone and overcoming spasm of the sphincter of Oddi, or to a reduction of edema of the ducts sufficient for causing their complete or partial occlusion. However, absence of bile does not mean that it fails to reach the duodenum at any time. Usually there is sufficient unaltered bile pigment to give the duodenal contents a tinge of yellow under normal conditions. A small amount of urobilin (a reduction product of bilirubin) is likewise normal but the chromogen (urobilinogen) is found only when urobilin is present in marked excess. Under the circumstances an excess of either or both has the same significance as their increase in the feces, being indicative of increased blood destruction in the hemolytic anemias or from other causes.

An examination of the duodenal contents, therefore, may be of practical value not only in relation to duodenitis, the diagnosis of gall tract disease (especially of cholelithiasis) and of increased blood destruction, but likewise in relation to the detection of achylia pancreatica due to chronic pancreatitis or other causes.

### COLLECTION

1. The Lyon tube is 130 cm. in length, with a pear-shaped metallic tip having an elongated grooved shank securing it to the tube without tying by thread, thereby minimizing trauma. This tube has two marks equidistant from the ends, and 20 cm. apart. The single mark at 55 cm. from the tip represents the average distance from the lips to the greater curvature of the stomach, and the double mark at 75 cm. to a position approximately at the level of the ampulla of Vater. To the outer end is attached a glass observation cannula or window, which, in turn, connects a larger size tubing, 30 to 50 cm. in length. Because the marks on this tube are equidistant from the ends, the tube may be reversed when wear and tear begin to appear on the swallowed portion. These tubes are also equipped with an adjustable rubber collar at the duodenal mark, which serves to record the variations in length effective for individual patients, and to enable the patient to feel its contact on the lips without difficulty.

Other useful tubes are those of Reh fuss, Twiss, Levin and Jutte (well adapted for use in infants).

2. Each patient is also provided with a tray containing a one-ounce capacity Asepto bulb syringe, a kidney basin, a clamp, a percolator or funnel, two 250 cc. graduates, and 125 cc. graduate, and three or more 250 cc. bottles fitted with perforated rubber stoppers with glass tubing inserted for the collection of bile.

3. *Stimulants for Bile Flow.*—(a) Saturated solution of magnesium sulfate diluted with two volumes of sterile water to make a 33 per cent volumetric solution. With this the patient is stimulated one or more times, depending upon the amount of magnesium sulfate solution that has been retained. The following fractional dose is advised: first stimulation of 45 cc., recovering as much as possible; second stimulation

of 30 cc.; third stimulation 30 cc. Care should be taken that not more than 90 cc. of the 33 per cent solution (equivalent to one ounce of the saturated solution) is retained at any one treatment, because of the danger of a severe adynamic ileus of the upper bowel. (b) Fifty to 100 cc. of a 5 per cent sterile solution of peptone (boiled and filtered). (c) Fifteen to 30 cc. of olive oil warmed to body temperature. Adding 15 cc. of hot water facilitates its delivery through the tube. Since the oil rises to the top of the specimens and does not dilute the flow of bile, it affords the most satisfactory results when quantitative chemical analyses are to be carried out. Olive oil frequently produces a more prompt flow of B-bile than occurs with magnesium sulfate, and occasionally will cause such a flow after previous drainages with the magnesium salt have failed to do so. The chief objection to the use of olive oil, however, is that it interferes with proper microscopy, especially at the hands of a beginner. (d) Five to 10 cc. of oleic acid, chemically pure, in 15 to 30 cc. of water.

4. *Preparation of the Patient.*—The most satisfactory time for doing a diagnostic drainage is in the morning on a fasting stomach. In preparation, the patient may be instructed to eat a meat sandwich, 20 raisins, and a glass of milk or water at 9 P.M. the previous night, as an optional motor test meal. The reason for this test meal is to introduce easily recognized food material so that if stasis occurs, it may be detected in the aspirates the following morning.



FIG. 108.—METHOD OF PASSING THE DUODENAL TUBE (SWALM)

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

After a 12-hour fast, drainage is then performed the following morning at 9 o'clock. Brushing of the teeth should be omitted to prevent the swallowing of blood from bleeding gums. A few moments of simple explanation as to the procedure generally gains the confidence of the patient. For the beginning of the drainage, the patient, if not too ill, sits on a chair, having removed any dentures or tight clothing.

5. *Intubation.*—(a) In passing the tube, stand a little to the right of the patient, facing her with the tip of the tube in the right hand (Fig. 108), and explain that when the tip is placed in the back of the mouth she should alternately swallow and breathe naturally through the nose, holding the head in a natural position, until the tip has passed the glottis, after which the tube may easily be slipped down to the stomach mark in the absence of esophageal obstruction or an excessive gag reflex.



FIG. 109.—METHOD OF GASTRIC LAVAGE IN DUODENAL DRAINAGE (SWALM)

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

(b) The fasting gastric residuum is now extracted by gravity. Only occasionally is the syringe needed to start the flow, but always with minimum suction to avoid trauma. This residue should be described and examined, especially for free and total hydrochloric acid, occult blood, bile, mucus, and detailed microscopy.

(c) The stomach is now washed with several 250 cc. units of sterile water at body temperature through a percolator or funnel placed about 18 inches above the patient's head. This is returned by syphonage into a graduate on the floor (Fig. 109).

(d) Following this, 100 cc. of sterile water may be introduced through the tube into the stomach to encourage gastric peristalsis in carrying the tip through the pyloric canal, and the tube is then clamped.

(e) The patient is instructed to lie on the bed on her right side (Fig. 110) and *slowly* swallow the tube, taking 20 minutes to get the duodenal mark on the lips. One minute for each centimeter on the Lyon tube will usually engage the tip synchronously with the frequent peristaltic waves which will carry it through the pyloric canal.



FIG. 110.—DUODENAL LAVAGE IN THE SIMS' POSITION (SWALM)

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

(f) The tube is then unclamped and connected to the first bottle. The first fluid to appear will be either pearly gray or yellow duodenal-pancreatic fluid. The fasting duodenum frequently already contains bile.

(g) In locating the position of the duodenal tip and assuring one's self that it is at the proper level, fluoroscopy is rarely necessary. If there exists any doubt as to the location of the tip, stethoscoping the abdomen for maximum air explosions over the stomach and duodenum with air introduced under syringe pressure through the tube will prove highly reliable. An experienced technician, however, can easily and quickly locate the position of the tip when using an Asepto bulb syringe, and, with an "educated thumb" constantly controlling the pressure on the bulb, by injecting a little water will find that if the tip is in the stomach, all fluid will return quickly; if in the pyloric canal, the water will go in very slowly and there will be no return of the fluid, but only a decided tug on the bulb; if in the duodenum, the water will enter slowly

and only a portion, generally bile-stained, will return. If the tube is not in the duodenum, it is then withdrawn well up in the stomach and slowly reswallowed. A tube buckled in the stomach or at the pyloric orifice may be partially withdrawn without any initial resistance, but if the tip is in the pylorus or duodenum a slight initial resistance is felt. Rarely a worm tube knots itself in the stomach.

6. *Stimulation for Bile Flow.*—(a) With the tip definitely in the duodenum, bile drainage is established by serial stimulations with one or more of the stimulants described above. These solutions should all be introduced at body temperature and the tube clamped for 2 to 5 minutes before syphonage is established. This will begin immediately provided the tube remains filled with fluid before being clamped. A mixture of magnesium sulphate and peptone will sometimes give better results than either alone, and both are satisfactory for bile microscopy and culture.

(b) Over a 3-hour drainage period the amount of bile mixture usually recovered by an *adequate drainage* will total 250 to 400 cc., including 20 cc. of pearly gray or yellow duodenal fluid (D-bile), 10 to 20 cc. of golden-yellow duct bile (A-bile), 30 to 75 cc. of dark yellow-brown, mahogany, dark green, or black gallbladder bile (B-bile), and at least 200 cc. of golden to lemon liver bile (C-bile). (See Plate V.)

(c) *Cultures* are made as desired from any bile sample. For gallbladder culture the best material is afforded by the last of the B-bile (the dregs from the floor of the gallbladder). This is theoretically so but is practically difficult to accomplish. Cultures are taken directly into glucose hormone bouillon in the special flasks devised by Richardson (Fig. 111). Or the bile may be collected in a sterile vial and plated in the laboratory.



FIG. 111.—SPECIAL CULTURE FLASK

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)

PLATE V



NORMAL A, B AND C FRACTIONS OF BILE

FIG. 1.—Common duct  
bile (chiefly).

FIG. 2.—Gallbladder  
bile (chiefly).

FIG. 3.—Liver bile  
(pure).

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)





(d) *Withdrawal of Tube*.—Before withdrawing the tube from the duodenum to the stomach and from the stomach to the esophagus, a little air should be blown through the tube to balloon the walls away from the metal tip to avoid scratching the mucous membrane. As the tube reaches the glottis, a swallowing movement will facilitate its withdrawal. The duodenum and stomach may be lavaged during removal as desired. A stomach wash with water during withdrawal to remove any regurgitant bile, and a mouth wash, a cup of broth, and some crackers after withdrawal add to the comfort of the patient. Tubes should be flushed out and sterilized by boiling after use. Good rubber should be used in the manufacture of these tubes.

*Sources of Difficulty*.—Occasionally difficulty may be experienced in getting the tip through the pylorus, or the tube may be sharply buckled in the stomach. In such a situation withdrawal of the tip well up into the fundus is necessary prior to reswallowing. The following conditions must be considered: Faulty technic; pylorospasm; gastropnoia and gastric atony; organic disease of the stomach and duodenum and accidents.

According to Swalm, if bile is not obtained at the first attempt, the procedure should be repeated after sedatives and antispasmodics have been administered orally for several days. In case of thick gastric mucus, spirits of ammonia and sodium bicarbonate,  $\frac{1}{2}$  dram of each, may be added to 250 cc. units. If after the tube reaches the duodenum, drainage does not proceed properly, it may be started by tilting upwards the glass connection, thus reducing air pressure. In stubborn cases, 1/100 grain of atropine in 25 cc. of warm water, clamped off for 5 minutes, will often facilitate the procedure. If duodenal mucus is found to be blocking the tube, lavage several times with 25 cc. magnesium sulfate in 225 cc. of warm water will usually overcome the difficulty.

In obstinate cases, in which there is difficulty in obtaining "B" bile after ordinary stimulation, the use of a mixture of peptone (1 dram in 75 cc. of water, boiled and filtered) with 25 cc. of magnesium sulfate solution and 1/100 grain of atropine will often succeed. Drainage in difficult cases can also be sometimes started by instructing the patient to rise on the elbow for 2 or 3 minutes while air is syringed in and out of the tube. In the case of nervous individuals it is advisable to give bromide and atropine orally on arising in the morning. Most patients relax better when given some light reading during the drainage.

## MACROSCOPIC EXAMINATIONS OF DUODENAL CONTENTS

1. A cloudy turbid fluid may be due to the admixture of acid stomach contents with a resulting precipitation of the bile salts (this turbidity disappears on neutralization with sodium hydroxide) or to the presence of bacteria and pus or duodenal cells. A turbid, alkaline fluid is indicative of a duodenitis or inflammation of the gall tract.

2. Normally only small amounts of mucus are present as occasional floccules. An excess may be due to duodenitis, in which case the flocculi, composed of mucus and epithelial cells, are not stained or only slightly stained by bile. If due to inflammation of the gall tract, the flocculi are usually bile-stained and contain columnar epithelial cells and leukocytes. In some cases of cholecystitis where there are many small calculi, the duodenal contents may show the presence of orange-yellow, coarse, putty-like detritus. Pure blood is rarely observed. Blood stained flocculi are often due to acute duodenitis or peptic ulcers.

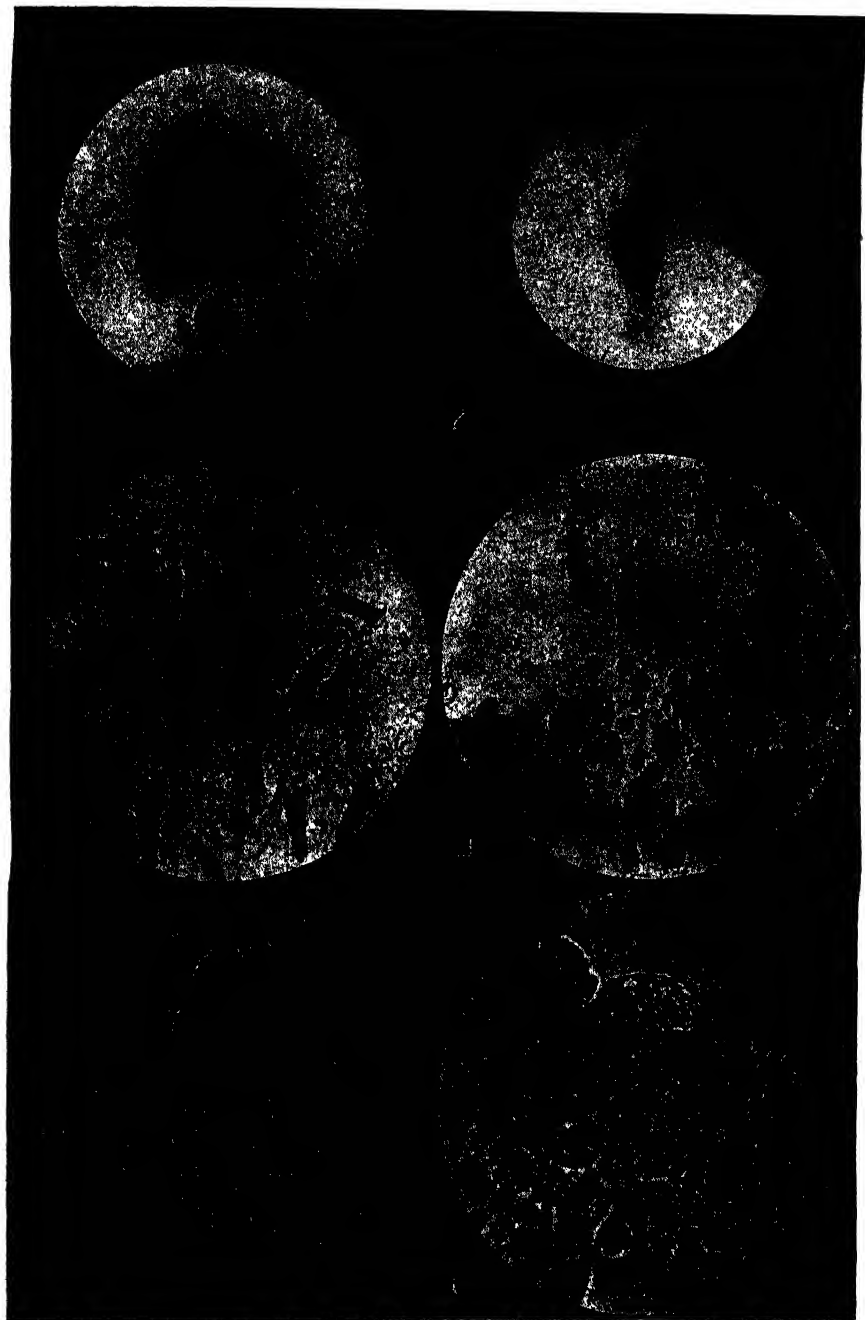


FIG. 112.—MICROSCOPY OF DUODENAL CONTENTS AND BILE

1, stratified pavement epithelial cells from buccal membrane of normal mouth,  $\times 350$ ; 2, isolated esophageal epithelial cells,  $\times 380$ ; 3, gastric cells with conspicuous degenerative changes,  $\times 360$ ; 4, duodenal cells, oval or cuboidal. Most common type in "wet" preparations,  $\times 380$ ; 5, gallbladder cells, bile stained,  $\times 380$ ; 6, gallbladder cells obtained on biliary drainage,  $\times 380$ . (From Lyon, *An Atlas on Biliary Drainage Microscopy*.)

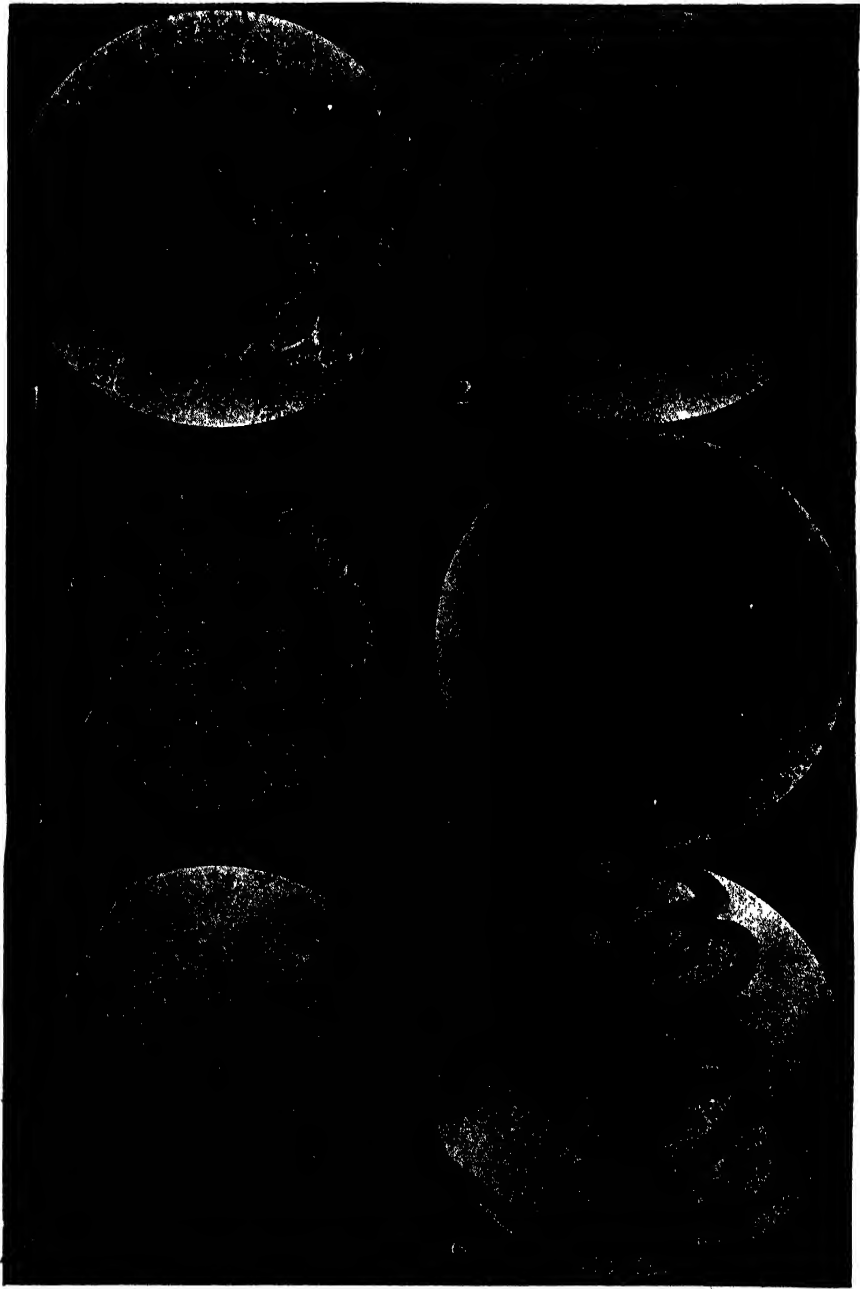


FIG. 113.—MICROSCOPY OF DUODENAL CONTENTS AND BILE

1, salivary corpuscles, oval or round cells containing irregular sized granules,  $\times 380$ ; 2, myelin threads from gastric tubules, rolled into small snail-like bodies by gastric peristalsis,  $\times 380$ ; 3, pus cells with intact protoplasm,  $\times 380$ ; 4, yeast, leptothrix and mixed bacteria,  $\times 250$ ; 5, vegetable forms of *Giardia lamblia*  $\times 460$ ; 6, larvae of *Strongyloides stercoralis*, Lugol's stain,  $\times 360$ . (From Lyon, *An Atlas on Biliary Drainage Microscopy*.)

**MICROSCOPIC EXAMINATIONS OF DUODENAL CONTENTS**

1. Select floccules of material from the sediment of the uncentrifuged specimen and prepare wet cover slip preparations. These examinations must be made at once, before any of the elements are digested or changed.

2. Examine for the following (Table 11): (a) *Epithelial cells*, which may be present from the mouth, esophagus, stomach, duodenum or gall tract (Fig. 112); (b) *salivary corpuscles* (Fig. 113); (c) *myelin threads* (Fig. 113); (d) *pus* (Fig. 113); (e) *bacteria* (Fig. 114); (f) *animal parasites* (Fig. 113); (g) *mucus* (Fig. 115); (h) *oleaginous substances* (Fig. 115); (i) *food remnants* (Fig. 115) and (j) *crystals* (Fig. 115).



FIG. 114.—BACTERIAL COLONY IN DUODENAL CONTENTS

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)

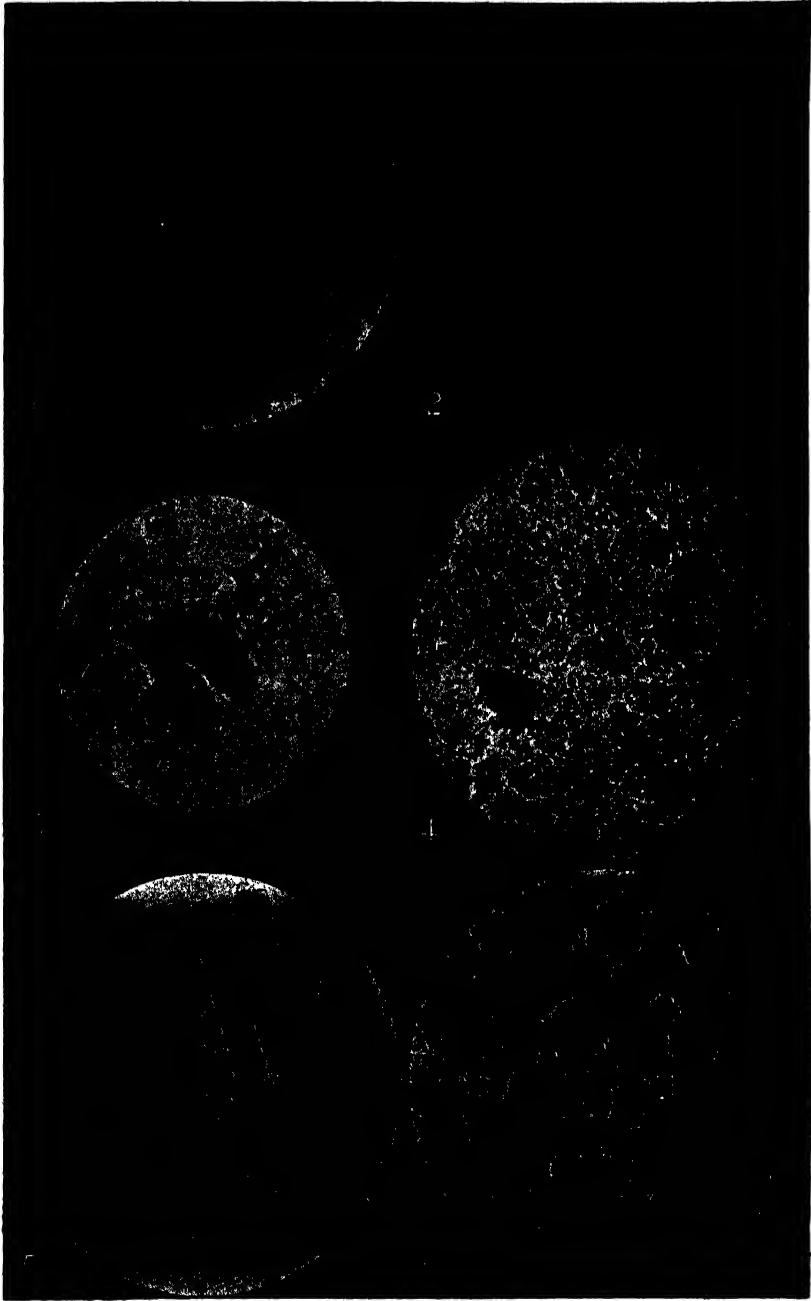
**CHEMICAL EXAMINATIONS OF DUODENAL CONTENTS**

**Trypsin Test.**—This test is based upon the principle that trypsin will digest fibrin liberating tyrosin and tryptophan.

1. Prepare fibrin by allowing animal blood to flow into a bowl. Rapidly whip with an egg beater made of a bundle of wires. Collect the fibrin and wash free from blood until it has a very light color. Preserve in glycerol or in water to which several cc. of chloroform have been added.

2. Test the duodenal fluid with litmus paper. If acid or neutral, slightly alkalinize with a few drops of 1 per cent aqueous solution of sodium carbonate.

3. In a test tube place 5 cc. of duodenal fluid, 1 gm. of fibrin and 2 drops of toluol. Incubate at 37° C. for 24 to 48 hours.



**FIG. 115.—MICROSCOPY OF DUODENAL CONTENTS AND BILE**

1, mucus ribbons or bands from duodenal zone in sheets,  $\times 180$ ; 2, grade one oleaginous degeneration,  $\times 380$ ; 3, combination of cholesterol and calcium bilirubinate crystals,  $\times 360$ ; 4, mixture of calcium, cholesterol and bile pigment crystals; 5, striated meat fiber,  $\times 200$ ; 6, starch cells from bread,  $\times 460$ . (From Lyon, *An Atlas on Biliary Drainage Microscopy*.)

4. Centrifuge the digestion mixture and examine the sediment microscopically for tyrosin crystals (Fig. 93).

5. To a small amount of the clear digestion mixture, add 1 drop of bromine water prepared by diluting 0.5 cc. of reagent bromine to 100 cc. with distilled water. Mix and watch for the appearance of a reddish-violet color. If no color develops, add another drop of bromine water. The color appearing, due to tryptophan, disappears on the addition of an excess of bromine water.

6. Tyrosin crystals and the tryptophan color reaction indicate the presence of trypsin in the duodenal fluid.

**Test for Amylopsin (Pancreatic Amylase).**—This test may be conducted with duodenal contents in the same manner as the test for amylase in saliva, described on page 180. Amylase is characteristically increased in the serum or plasma in acute pancreatitis and in carcinoma of the pancreas.

**Test for Steapsin (Pancreatic Lipase).**—This test is based upon the principle that lipase will digest the fat of milk, liberating fatty acids which impart an acid reaction to litmus.

1. Test the duodenal fluid with litmus paper. If acid or neutral, slightly alkalinize with a few drops of 1 per cent aqueous solution of sodium carbonate.

2. To fresh milk add sufficient litmus solution to give a distinct blue color and place 10 cc. in each of 2 test tubes.

3. Boil 5 cc. of duodenal fluid, cool and add to 1 tube of litmus milk. Add 5 cc. of unheated duodenal fluid to the second tube. Place both tubes in a water bath at 37° C. for 15 to 30 minutes, or longer if required.

4. Note the color of the tubes. A red color in the second tube containing the unheated duodenal fluid with no color change in the first tube indicates the presence of lipase. A red color in both tubes indicates that at least part of the acidity is due to causes other than lipolytic activity.

**Einhorn's Method for Trypsin, Amylase and Lipase.**—1. Prepare capillary tubes of hemoglobin by rubbing 1 gm. of hemoglobin powder in 10 cc. of distilled water until a smooth paste is obtained. Add 2.5 gm. of powdered agar and 90 cc. of distilled water. Place in a flask and heat to boiling. Draw by suction while still hot into warm capillary glass tubing (inside diameter about 1.5 millimeter). After cooling, cut into small pieces (3 centimeters length) and seal ends with paraffin.

2. Prepare capillary tubes of starch in the same manner, using 5 gms. of starch, 2.5 gms. of powdered agar and 100 cc. of distilled water. Keep in refrigerator.

3. Prepare capillary tubes of olive oil by rubbing together 25 cc. of olive oil and 2 gms. of powdered agar to make a thin paste and then add 100 cc. of a 1 : 2000 aqueous solution of Nile blue sulfate. Mix thoroughly, heat to boiling and fill capillary tubes as described. Keep in refrigerator.

4. Place 5 cc. of fresh unfiltered duodenal fluid in a test tube. File off the ends of a hemoglobin, starch and olive oil capillary tube (avoid dried out ends). Place the capillary tubes into the test tube with open ends down.

5. Place the test tube in an incubator at 37° C. for 24 hours.

6. Examine the 2 ends of the hemoglobin tube for clear, digested area; measure each with millimeter rule; take the average and report in numbers of millimeters. The digestion is due to the presence of trypsin.

7. Examination of starch tube. Push the contents of tube out on a slide and cover

with Lugol's solution; measure the part which does not turn blue as above and report in millimeters. The digested portion fails to give the starch reaction with iodine. The digestion is due to the presence of amylase.

8. Examine the ends of the olive oil tube and measure the portion which has turned violet. The Nile blue in the digested portion turns violet. Report in number of millimeters as above. The digestion is due to the presence of lipase.

### MACROSCOPIC EXAMINATIONS OF BILE

The gross description of each of the bile fractions should include:

1. *Amount*.

2. *Color*. Red and green biles are anomalous and may be obtained at times directly from the liver or gallbladder. The admixture of gastric juice will frequently cause any specimen to turn green on standing.

3. *Flocculation and precipitation*. The amount of white or bile-stained flakes suspended in or setting on the bottom of the receiving bottle in *uncentrifuged* specimens should be recorded as to degree, as follows:

+ = occasional floccule (normal).

++ to +++++ = pathological.

4. *Blood* (pure blood rarely observed).

5. *Red mucous flecks* (often associated with gastric or duodenal ulcer).

6. *Viscosity* (normally slightly viscous, due to mucin).

As a rule it may be said that normal duct, gallbladder, and liver biles will be transparent and free from turbidity, except as occasional zones of turbidity, like an egg yolk emulsion, form in the drainage bottles as spurts of acid gastric juice (physiological action of the vagus) blend with the bile. This turbidity, caused by the precipitation of bile acids, will clear up on shaking the specimen if the pH of the duodenal bile mixture is sufficiently alkaline. Otherwise turbid biles are to be construed as pathological, especially when they contain much flocculent debris or sediment abundant in exfoliated epithelial cells, pus cells, bacteria, mucus, crystalline elements, or oleaginous substance.

Not infrequently in the presence of a non-functioning gallbladder, liver bile will be darker and more concentrated than normal. This concentration of bile may readily be ascertained by determining its bilirubin content, especially if olive oil has been used as the stimulant. The normal bilirubin content of liver bile, obtained by the use of olive oil, ranges from 2 to 10 mgm. per 100 cc. in the duodenal fluid, whereas the bilirubin content of abnormal liver bile ranges from 11 to 40 mgm. per 100 cc. when obtained under similar conditions. The "B" fraction, which is a mixture of gallbladder and liver bile, should exhibit an appreciable elevation in bilirubin content (15 to 80 or more mgm. per 100 cc.) if the gallbladder is functioning properly.

### MICROSCOPIC EXAMINATIONS OF BILE

1. Do not centrifuge the specimens.

2. Fish for desired floccules and sediments with a pipet and prepare wet preparations.

3. Examine fresh unstained wet preparations under both low and high dry magnification.



4. Examine at least three preparations from each specimen showing flocculation.
5. Examine for the following (see Table 11): (a) *Gallbladder epithelium* (Figs. 112, 116, 117 and 118); (b) *pus cells* (Fig. 113); (c) *mucus* (Fig. 115); (d) *oleaginous substances* (Fig. 115); (e) *crystals* (Figs. 115 and 119) and (f) *animal parasites* (Fig. 113).



FIG. 116.—SHORT COLUMNAR BILE-STAINED EPITHELIUM

(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)



FIG. 117.—TALL COLUMNAR BILE-STAINED EPITHELIUM

From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)



FIG. 118.—DEGENERATED BILE-STAINED COLUMNAR EPITHELIUM  
(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)

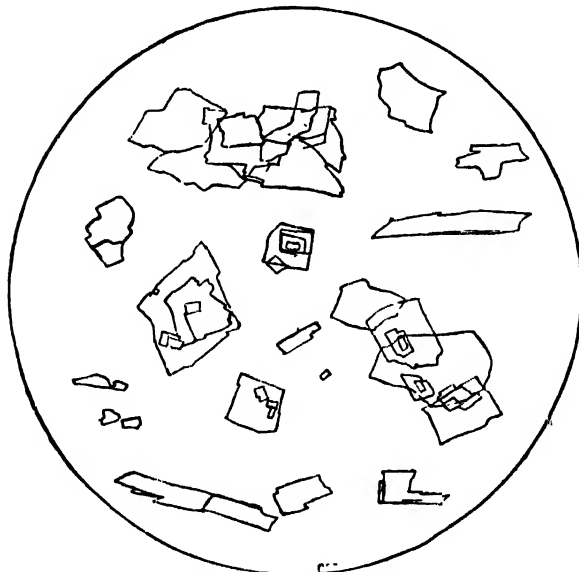


FIG. 119.—CHOLESTERIN CRYSTALS  
(From Lyon, *Non-Surgical Drainage of the Gall Tract*, Lea and Febiger, Philadelphia.)

### CHEMICAL EXAMINATIONS OF THE BILE

Much added information is needed on the chemistry of bile obtained by duodenal drainage in relation to clinical diagnosis. That significant changes occur in the gallbladder bile in disease has been shown by Ravdin<sup>2</sup> and others with bile chiefly obtained at operation, largely referable to changes in pH concentration, basic ions and chlorides. But few similar studies have been made with bile obtained preoperatively by duodenal drainage. The normal bilirubin of liver or "C" bile obtained by this method ranges from 2 to 10 mg. per 100 cc., whereas, the dark bile of gall tract disease ranges from 11 to 40 mg. per 100 cc. Chiray and Marcotte<sup>3</sup> have observed that in non-calculous cholecystitis the concentration of cholesterol and bilirubin is high, whereas when the gallbladder contains stones with a patulous cystic duct, the reverse obtains. However, it is necessary that studies on the chemistry of biles obtained preoperatively by duodenal drainage be compared with those obtained at operation on the same patients before any definite statements can be made concerning their diagnostic value. At the present time such studies by Shay and Riegel<sup>4</sup> have indicated that a close correlation has not been obtained, either in respect to chemical changes, or in connection with the results of cholecystography.

Insofar as normal bile obtained at operation or postmortem is concerned, chemical analyses have shown 8 to 18 per cent total solids, 1 to 4 per cent mucin and from 0.5 to 1.1 per cent inorganic material. But values outside of these limits are not uncommon. Hepatic duct bile is alkaline (pH 7.4 to 8.5) while gallbladder bile is slightly acid or neutral (pH 5.4 to 6.9). The chief ingredients are the bile acids in combination as alkaline salts, bile pigments, lipids, including cholesterol, mucoprotein and electrolytes (approximately equivalent to that of plasma in the case of liver bile while higher in gallbladder bile). The concentration of cholesterol in liver bile is less than that of the blood, most values being below 100 mg. per 100 cc. Gallbladder bile, however, contains more cholesterol (150 to 200 mg. per 100 cc.) and particularly in diseases of the liver and gall tract.<sup>5</sup>

### BAACTERIOLOGICAL EXAMINATIONS OF BILE

1. Smears of unstained and stained material may be examined for preliminary data as described above.
2. Cultures should not be made if there is excessive retching and gagging because of the chance of contamination.
3. Cultures are indicated (a) when large numbers of organisms are seen in smears; (b) in cases requiring a careful search for foci of infection, (c) in cases of obvious gallbladder infection in which vaccine therapy is to be tried.
4. The technic is described and bacteriological findings are given on pages 416 to 417.

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TABLE 11 \*

Object	Description	Cause of Excess	Comment
Oral mucous membrane	Stratified squamous epithelium	Stomatitis	May be accompanied by pus cells
Salivary corpuscles	4 to 5 times size of leukocyte: twice size of duodenal cell: oval or round, containing granules of irregular sizes		Rarely reach duodenum. May liberate granules as irregular, dense translucent gray bodies, probably lecithin, mucin, or myelin. Lecithin-like bodies at times found in bile.
Esophageal cells	Stratified squamous epithelium, with tendency to more narrow diamond-shaped pattern in deeper layers. More variation in size and shape of nucleus.	Simple esophagitis due to mucosal irritants: cardiospasm: cancer of esophagus: diverticulum.	Patients present deglutition difficulties
Myelin threads	Small snail-like bodies	Hyperperistalsis: high gastric acidity	Probably pressed out of gastric tubules: may be mucin, or derived from saliva
Pus cells	Nucleus prominent when cytoplasm digested by normal or increased HCl: not digested if hypochlorhydria exists. Bile-stained pus cell suggests biliary tract inflammation	Inflammation in zone where found or in adjacent zone above	Nuclei resist gastric digestion, but are readily attacked by pancreatic trypsin
Gastric cells	(a) Tall columnar cell predominates: goblets with biforked tails: usually not bile-stained (b) Columnar, but scanty, degenerated, thick-bodied, and hyalin in appearance	(a) Irritants  (b) Usually in chronic progressive atrophic gastritis	Oval and cuboidal cells of gastric epithelium do not exfoliate to appreciable degree
Duodenal cells	(a) Oval or cuboidal cell with single nucleus: often twice size of leukocyte: pearly gray unless bile-stained when devitalized (b) Same, with degenerative changes of hyaline and amyloid character, and increased in thickness and density: vacuolization and inclusion bodies in nucleus and cytoplasm (c) Atypical cell: larger and resembles resting amoeba, with granules bunched in cell, leaving small zone of clear cytoplasm	(a) Duodenitis  (b) Chronic duodenal disease  (c) Deep-seated duodenal ulcer: cancer of pancreas eroding through duodenal wall	Exact origin and nature still obscure, but represents the typical cell of the duodenum   (c) Phagocytic cells? Possibly derived from pancreas

\* Compiled from Lyon, *Atlas of Biliary Drainage Microscopy*.

Object	Description	Cause of Excess	Comment
Gallbladder cells	Single layers of simple tall columnar epithelium below which lie collections of polygonal cells: arranged in rows, fans, or rosettes. Bile-stained if devitalized	Cholecystitis: antedates all other diagnostic signs	"Polygonal" cells may sometimes be columnar cells viewed on end. Short columnar epithelium may be derived from duct epithelium
Micro-organisms	(a) Bile-stained bacterial colonies (b) Diffuse bacteria (c) Leptothrix-fungus-yeast group (d) Algae or diatoms	(a) Infection of biliary tract  (c) Secondary invaders (d) Seasonable, from water supply	(a) must be proved viable by culture
Mucus	(a) Duodenal mucus appears as well defined ribbons or bands: not necessarily bile-stained: may be tinged rusty red from hemoglobin products (b) Twisted or spiral mucus: invariably bile-stained: often dense and occasionally encrusted with granular deposits of brilliant yellow pigment or (?) bile acids. (c) Grossly visible mucus plugs	(a) Catarrh. Red mucus associated with duodenitis, erosion, or ulcer  (b) Catarrh of ducts, especially cystic duct  (c) from ducts	(a) Duodenal cells accompanying duodenal mucus are usually bile-stained  (b) Conformation of Heisterian valve would cause twisting  (c) Presence may check recession of catarrhal jaundice at times
Oleaginous substance	Yellow oily fluid as droplets, pools, and lakes: may melt out from unusually dense twisted and bile-stained mucus. May appear as amorphous bright yellow or orange substance, from which pools and lakes emerge on standing	Catarrh of bile ducts, especially of cystic duct when present with twisted mucus: also occurs occasionally with cholesterosis of gallbladder	Probable fatty ester of cholesterol: may form actual cholesterol crystals. May be associated with cystic duct occlusion until expelled. Olive oil droplets are colorless under the microscope.
Crystals	(a) Cholesterol plates: flat, colorless, thin fragments with chipped edges like splintered window glass  (b) Calcium bilirubinate: clusters of lustrous bright yellow, lemon, or orange finely or coarsely granular crystals: lustrous brilliancy differentiates from bile acids (c) Calcium: dirty grayish white, dense, thick, chunky	All crystals imply formed or forming calculi. They may also be a component of gall sand which drains off	(a) Especially significant when the more imperfect in form, or when associated with calcium bilirubinate  (b) The most gritty of all the biliary crystals under the cover slip  (c) Like other crystals, must be differentiated from vari-

Object	Description	Cause of Excess	Comment
Parasites	and irregular in size and shape		ous artefacts, such as dust particles, sterilizer rust, or rubber flecks from old tubes
	(d) Bile pigment: reddish amber to brown or black, with occasional reddish or yellow tints on thinned edge		
	(e) Tyrosine: long slender needles		(e) Should be differentiated from fatty acid crystals
	(a) Giardia: usually attached to ribbons of duodenal mucus or to duodenal cells by their sucker-like peristomes: dumbbell-shaped nucleus resembles pair of eye: pear-shaped with eight flagellae arranged in pairs: average 12-20 by 8-12 micra: resemble bowl of spoon when viewed on side: more refractile than cells: tumble and gyrate when motile: stunned by action of magnesium sulphate	(a) Infestation: potential pathogens	(a) Fairly common: transmitted by cysts from carriers handling fruits, vegetables, or water supply. Often found in enormous numbers. Resistant to treatment.
	(b) Strongyloides stercoralis larvae: actively motile	(b) Infestation	(b) Relatively rare except in tropics
Food	(c) Various rarer parasites (entameba histolytica, clo norchis sinensis, distoma hepaticum, chilomastix mesnili, ankylostoma duodenale, trichomonas and cercomonas hominis, ascaris lumbricoides)	(c) Infestation	(c) Ascaris worms may be vomited as a result of stimulation of gag reflex
	(a) Starch cells from bread: oval and spherical bodies: some closely resemble tenia ova	Excess indicates stasis and desirability of x-ray studies when found in fasting stomach or duodenum	For further description of microscopic food particles consult Lyon's Atlas.
	(b) Canned peas: might be confused with ova of diphyllbothrium latum		
	(c) Lima beans: might be mistaken for ascaris ova		

## METHODS FOR CONDUCTING LIVER FUNCTION TESTS

**Principles.**—1. No other organ has as many functions to perform as the liver. Indeed, its role in maintaining a constant supply of utilizable food materials requires its participation in every phase of metabolism, so that it has been aptly described by Mann as the “commissariat of the body”. Furthermore, it is the site of many synthesis as well as being an organ concerned in detoxication, secretion and excretion. Owing, however, to its large size, enormous reserve capacity, and remarkable regenerative powers, it has been estimated that about 75 per cent must be destroyed functionally or anatomically before gross impairment of its functions occurs. For this reason and because of its multiple functions, no one test for functional capacity has been found satisfactory.

2. Under the conditions, it is frequently impossible to detect evidences of dysfunction even in the presence of advanced anatomical damage, the result of either primary disease of the liver, or secondary to lesions of the gallbladder and extrahepatic ducts. This is especially true of chronic diseases such as cirrhosis, syphilis, carcinoma, etc., which, because of their slow progress, are associated with compensatory regeneration with little or no impairment of functions. In acute hepatic disease, however, and especially in hepatocellular necrosis, the functional tests yield more satisfactory results. Then, too, some of the functions of the liver are so intimately associated with other organs that it is difficult or impossible, as stated by Mann, to definitely determine the hepatic factor.

3. In view of the multiplicity of the functions of the liver it is apparent, therefore, that many laboratory examinations may reveal the presence of damage of this organ without being necessarily function tests. Among these may be mentioned various blood chemistry determinations as those for fasting blood sugar, amino acids, urea nitrogen, uric acid, fibrinogen, total proteins (with the albumin-globulin ratio), prothrombin, bilirubin, cholesterol and cholesterol esters and phosphatase, of fats and bile pigments in the feces and of urobilinogen in the feces and urine described herewith. Indeed, when any of these indicate liver damage under circumstances where nephritis and other extrahepatic disease may be excluded, function tests may not be required. But, otherwise and especially when it is important to estimate the degree of liver damage, the function tests may be advisable.

4. A large number of liver function tests have been proposed. Some, like the glucose and galactose tests are based upon the assumption that disturbances of the liver in relation to carbohydrate metabolism are sufficient for indicating dysfunction of this organ as a whole, while the Takata-Ara test has been proposed as a test for the detection of disturbances in the formation of plasma proteins (especially of globulins) in relation to protein metabolism and that of Hanger by noting the capacity of blood serum to flocculate a colloidal suspension of cephalin-cholesterol. The bilirubin tolerance test was designed for the detection of disturbances of pigment metabolism while the hippuric acid synthesis test was designed to detect disturbances in the detoxifying and conjugation functions. Others, like the bromsulphalein test, are based upon the assumption that the ability of the liver to excrete dyes is a sufficient measure of its functional capacity for clinical purposes.

**SPARKMAN'S TESTS FOR UROBILINOGEN IN THE FECES AND URINE**

**Principles.**—These tests depend on the so-called aldehyde reactions, with the development of a red color on the addition of an acid solution of paradimethylaminobenzaldehyde (Ehrlich's aldehyde reagent) to solutions containing urobilinogen. Comparison is made with artificial standards prepared from gold chloride and sodium bromide.

**Reagents and Standards.**—1. Prepare the aldehyde reagent by dissolving 10 gm. of paradimethylaminobenzaldehyde in 75 cc. of hydrochloric acid diluted with 75 cc. of distilled water.

2. Prepare a 4 per cent solution of gold chloride in distilled water.

3. Prepare a 10 per cent solution of sodium bromide in distilled water.

4. Prepare a *strong standard* by diluting 1 volume of sodium bromide solution and 1 volume of gold chloride solution with 13 volumes of distilled water. The computed final value is equivalent to 8.2 mg. urobilinogen per 100 cc.

5. Prepare an *intermediate standard* by diluting 1 volume of strong standard with 1 volume of distilled water. The computed final volume is equivalent to 2.4 mg. urobilinogen per 100 cc.

6. Prepare a *weak standard* by diluting 1 volume of intermediate standard with 1 volume of distilled water. The computed final value is equivalent to 0.9 mg. urobilinogen per 100 cc.

**Procedure for Fecal Urobilinogen.**—1. Transfer 5 gm. of the mixed stool to a mortar, emulsify in 100 cc. distilled water, and transfer to a 250 cc. Erlenmeyer flask.

2. Dissolve 8 gm. crystalline ferrous sulfate in 40 cc. of water and mix with emulsion.

3. Add slowly 40 cc. of 10 per cent sodium hydroxide, with rotation of flask. Stopper and shake.

4. Place in water bath for 15 minutes at 50° C. Cool to room temperature, then filter (Whatman No. 2).

5. Pipet 5 cc. of filtrate into test tube. Add 5 cc. distilled water, 0.3 cc. of five times normal hydrochloric acid, and 1 cc. aldehyde reagent. Allow 5 minutes for full development of color.

6. Compare in colorimeter with nearest standard and calculate as follows:

(1) *Strong standard:*

$$\frac{\text{Reading Standard}}{\text{Reading Unknown}} \times 630 = \text{mg. of urobilinogen per 100 gm. of stool.}$$

(2) *Intermediate standard:*

$$\frac{RS}{RU} \times 185 = \text{mg. urobilinogen per 100 gm. of stool.}$$

(3) *Weak standard:*

$$\frac{RS}{RU} \times 70 = \text{mg. urobilinogen per 100 gm. of stool.}$$

**Procedure for Urinary Urobilinogen.**—Single samples should be freshly voided. Twenty-four-hour specimens are collected in brown glass bottles containing 100 cc. purified petroleum benzine and 5 gm. anhydrous sodium carbonate.



1. To a small portion of urine in a flask, add anhydrous calcium chloride in the proportion of 50 cc. of urine to 2 gm. of the salt. Mix well and filter.

2. Test residue for bile pigment. Pour a few drops of concentrated nitric acid down side of filter. A colored zone consisting of a central pink area with a green periphery indicates the presence of bilirubin.

3. To 10 cc. of filtrate add 1 cc. of aldehyde reagent and invert several times. Allow 5 minutes for full development of color.

4. Match sample promptly in colorimeter. If concentrate is unusually high, repeat procedure after suitable dilution of the specimen and calculate as follows:

(1) *Strong standard:*

$$\frac{R \text{ Standard}}{R \text{ Unknown}} \times 8.2 = \text{mg. per 100 cc. of urine.}$$

(2) *Intermediate standard:*

$$\frac{RS}{RU} \times 2.4 = \text{mg. per 100 cc. of urine.}$$

(3) *Weak standard:*

$$\frac{RS}{RU} \times 0.9 = \text{mg. per 100 cc. of urine.}$$

**Interpretation.**—Normal values given by Sparkman are between 150 and 300 mg. of urobilinogen per 100 gm. of stool, with extreme limits of 70 to 600 mg. Any specimen of urine yielding a color in the range of the weak or intermediate standard may be regarded as not containing increased amounts of urobilinogen, and should be reported as “not increased”. Colors in the range of the strong standard should be regarded with suspicion. Values of over 8 mg. per 100 cc. of urine almost certainly represent pathological urobilinogenuria:

	Urinary Urobilinogen	Fecal Urobilinogen
Obstructive jaundice .....	Not increased	Decreased
Hepatogenous jaundice .....	Not increased	Great increase
(without liver cell disease)		
Portal cirrhosis, hepatitis, anemia .....	Decidedly increased	Decreased
(liver cells affected)		
Catarrhal jaundice .....	Early increased, later decreased (intrahe- patic obstruction). then increased.	Decreased at height of disease

**GLUCOSE TOLERANCE TEST**

**Principle.**—This test is based upon the failure of storage of glycogen in the liver with increased or prolonged hepatic glycogenolysis in liver disease.

**Procedure.**—1. Collect a specimen of venous blood in oxalate 12 hours after last meal (preferably before breakfast).

2. Administer 50 to 100 gms. of glucose in 250 to 500 cc. of cold water flavored with lemon.

3. Remove specimens of blood  $\frac{1}{2}$ , 1, 2 and 3 hours later.

4. Advisable to also collect arterial (capillary) blood before and at the same intervals after the administration of the glucose.

5. Determine the glucose content of each specimen of blood.

6. Normally the venous blood glucose reaches 140 to 160 mg. per 100 cc. within an hour returning to about the fasting level in  $2\frac{1}{2}$  hours. Arterial (capillary) blood glucose increases more rapidly and is from 10 to 15 mg. higher with a slower return to normal.

7. Hepatic dysfunction is indicated by a higher venous blood glucose within 1 to 3 hours with a rapid fall. The difference between venous and arterial (capillary) blood glucose is normal or but slightly different.

**GALACTOSE TOLERANCE TEST**

**Principle.**—This is a test for the efficiency of glycogen storage. Galactose is not a renal threshold substance.

**Procedure.**—1. Collect blood (oxalate) and urine before breakfast.

2. Administer 1 gm. of galactose per kilo of weight (usually 50 gm.) dissolved in 250 to 500 cc. of cold water flavored with lemon.

3. Collect urine and blood hourly for 5 hours thereafter. Water may be allowed but no food.

4. Determine the sugar content of each specimen of blood.

5. Test each specimen of urine for sugar by the Benedict qualitative method.

6. Mix the specimens giving positive reactions, measure and determine the total amount of sugar excreted by the Benedict quantitative method.

7. The test may be conducted also as follows: (a) Measure the total volume of the urine samples in cc.

(b) Divide this volume by 100 and with a Mohr pipet transfer this amount to a 250 cc. cylinder. Dilute carefully with distilled water to 150 cc. and mix thoroughly.

(c) Determine the amount of sugar present in 2 cc. of the diluted urine by the Benedict quantitative method. As galactose gives only 80 per cent as much reduction as glucose, the result must be multiplied by 1.25 to obtain galactose. If the Duboscq colorimeter is used, set the unknown at 25 and calculate as follows:

$$\frac{7.5S}{100} = \text{grams of galactose in total specimen}$$

When the fasting urine contains sugar, or it is desired to perform the test on a diabetic patient, a portion of the urine should be fermented with washed yeast sus-

pension. Consider carefully the dilution of the urine or use other appropriate calculations.

8. Normally the total elimination of sugar in the urine is not usually above 3 gm. although less than 6 gm. may not be significant. The blood sugar in the 2-hour specimen is not usually more than 30 to 120 mg. per 100 cc.

9. A total of 6 gm. or more in the urine is usually indicative of serious hepatic injury.

### BILIRUBIN TOLERANCE TEST

**Principles.**—This test proposed by Eilbott, has its greatest field of usefulness in cases of liver disease in which the serum bilirubin does not exceed 1 mg. per 100 cc. Soffer<sup>1</sup> believes that it may be the most sensitive single function test of all but it has the drawbacks of high cost of bilirubin and the analytical precision essential for eliciting small differences, especially when the total amount in the blood is small. It is based upon the excretion of bilirubin by the parenchymal cells of the liver.

**Procedure.**—1. Prepare a solution of chemically pure bilirubin by dissolving 1 mg. per kilogram of body weight (should not exceed 70 mg.) in 15 cc. of a 1/10 molar solution of sodium carbonate which has been previously brought to the boiling point and allowed to cool to 80° C.

2. Take a sample of venous blood in oxalate (preferably in a paraffined tube) with a dry syringe and then inject the solution of bilirubin through the same needle.

3. Obtain samples of blood in the same manner from the other arm 5 minutes and again 4 hours later. Allow no food or water.

4. Estimate the bilirubin in each sample of blood. In conducting these tests 2 cc. amounts of plasma of the control and 4-hour samples are thoroughly shaken with 2 cc. of redistilled acetone, while 1 cc. of plasma of the 5-minute sample is thoroughly shaken with 4 cc. of acetone. The plasma and acetone mixtures are then centrifuged and filtered directly into a dry microcolorimeter cup and compared with a standard 1:6000 solution of potassium bichromate. The difference in bilirubin concentration between the first 2 specimens is taken to represent 100 per cent of the injected pigment and is the basis upon which is computed the per cent retention at the end of 4 hours.

5. Normally all or nearly all of the bilirubin is excreted in 4 hours and the blood does not show a retention above 6 per cent at the highest.

6. A retention of more than 6 per cent indicates hepatic impairment.

### QUICK'S HIPPURIC ACID SYNTHESIS TEST

**Principles.**—The investigations of Quick<sup>2</sup> have shown that the liver is perhaps the principal site of the formation of hippuric acid by the conjunction of glycine and benzoic acid with its elimination in the urine. In certain types of hepatic disease this function is impaired due primarily to a diminished capacity of the liver to synthesize glycine, which is essential for the formation of hippuric acid, and in part to damage of the enzymatic mechanism which unites benzoic acid with glycine. The urinary elimination of hippuric acid after the ingestion of benzoic acid is, therefore, regarded as a measure of the capacity of the liver to furnish glycine (amino-acetic acid) and as an index of its detoxifying activity as well.

**Procedure.**—1. In conducting the *oral test* give 5.9 gm. of sodium benzoate dissolved in 30 cc. of water (flavored with a teaspoonful of cherry syrup) 1 hour after breakfast (coffee and toast). In the same glass (to be certain that all of the solution is taken) give  $\frac{1}{2}$  glass of water.

2. Immediately thereafter have the patient void urine and discard the specimen. Thereafter collect urine at hourly intervals for 4 hours and mix.

3. In conducting the *intravenous test* in the case of patients who are nauseated or vomiting or who are too ill to be deprived of fluids for the oral test give a breakfast of toast and coffee. If the patient is vomiting, or cannot take solid food, the entire meal, or the toast may be omitted. One hour later have the patient void urine and inject intravenously 20 cc. of a sterile aqueous solution containing 1.77 gm. of sodium benzoate. One hour after the completion of the injection collect urine (by catheterization if necessary).

4. If the urine samples contain protein, this must be completely removed before the determination of the hippuric acid. Measure the volume of the sample, add 5 cc. of 20 per cent copper sulfate solution and mix. With agitation, introduce 5 cc. of normal sodium hydroxide solution per 100 cc. of urine. In exceptional cases where there is a very large albumin content, double these amounts. Shake and heat to near the boiling temperature. After cooling, filter through a good grade of filter paper into a graduated cylinder and record the volume of the clear filtrate. Then proceed as for normal urines. In the calculations, take into account the fact that the urine was diluted with the protein precipitants and only an aliquot portion was used for analysis.

5. Measure the volume of urine. If over 150 cc., add a few drops of glacial acetic acid and evaporate to 150 cc. If less, note the volume. Add 30 grams of sodium chloride for each 100 cc. of urine and heat with shaking until the salt is dissolved. Cool the solution to 15 to 20° C. by immersion in ice-cold water. Then add 1.2 cc. of approximately N/10 sulfuric acid and scratch the insides of the flask with a glass rod to enhance the crystallization of the hippuric acid. (This step is important.) Allow to stand for 15 minutes in the cold water-bath and then filter through a Hirsch funnel (diameter of perforated plate, 47 mm.), using moderate suction. Wash the precipitate with chilled 30 per cent sodium chloride solution from a wash bottle, using the washing fluid first to rinse the flask in which the precipitation has been performed. It is not necessary to transfer the precipitate to the filter quantitatively. The precipitate is adequately washed when the washing fluid is free of sulfuric acid. Transfer the funnel with its contents to the flask that still contains some of the hippuric acid crystals, and rinse the filtered hippuric acid into the flask by dissolving in hot water, using a fine tipped wash bottle. All of the hippuric acid is now in the flask in which it was precipitated. Heat the flask until the hippuric acid particles adhering to the flask dissolve and titrate while hot with five-tenths normal sodium hydroxide using phenolphthalein as an indicator.

The cc. of five-tenths normal sodium hydroxide solution used for neutralization  $\times 0.072 =$  the amount of sodium benzoate in grams from which the hippuric acid was derived. To this amount add the correction for the solubility of hippuric acid in terms of sodium benzoate, *i.e.*, 0.10 gram of sodium benzoate for each 100 cc. of urine. The result is the sodium benzoate value of the specimen with a maximum error of  $\pm 10.5$  mg. per 100 cc.

**Example:** The volume of a urine specimen analyzed was 134 cc. The amount of

sodium chloride added was  $1.34 \times 30 = 40.2$  grams. The precipitated hippuric acid required 11.2 cc. of five-tenths normal sodium hydroxide solution for its neutralization. This corresponds to  $11.2 \times 0.072 = 0.806$  grams of sodium benzoate. The correction for solubility would be  $1.34 \times 0.10 = 0.134$  gram of sodium benzoate. The sodium benzoate value of the specimen, therefore, was  $0.806 + 0.134 = 0.940$  gram or 940 mg. The probable error is  $\pm 10.5 \times 1.34 = \pm 14$  mg. of sodium benzoate, which corresponds to 1.5 per cent of the quantity determined.

Occasionally one encounters urine specimens that are markedly bile-tinged or dark-colored. The analysis of such urines is difficult. Add 0.3 gm. of acid-washed norit per 100 cc. of urine and boil the mixture for about 1 minute. After cooling, filter by suction on a Hirsch funnel, and wash the norit residue with a small quantity of hot water. Measure the volume of the filtrate and proceed as outlined above.

6. In the *oral* test the normal elimination of hippuric acid is 3 to 3.5 gms. in the total 4-hour urine. In the *intravenous* test the total excretion is about 0.7 gm. in the total 1-hour specimen of urine.

### BROMSULFALEIN TEST

**Principles.**—This test is based upon the excretion of bromsulfalein by the parenchymal cells of the liver aided by destruction of the dye by the Kupffer cells. Some may also undergo destruction in the tissues. The test is invariably positive, and readings are unsatisfactory, when marked jaundice is present. Since the dye is expensive, it is suggested that its use be dispensed with if an appreciable degree of jaundice exists.

**Procedure.**—1. Weigh the patient and calculate the dose required, allowing 5 mg. for each kilogram of weight. The dye is available in a 5 per cent solution of which 0.1 cc. contains 5 mg. (Hynson, Westcott, and Dunning) so that the dose is 1 cc. for each 10 kilograms, equivalent to 22 pounds of body weight.

2. Withdraw 5 cc. of blood in a dry test tube as a control and then *slowly* inject the dye through the same needle with due care against extravascular injection.

3. At the end of 30 minutes and again 1 hour after the injection secure 5 cc. of blood from the other arm, using a dry needle and syringe to avoid hemolysis.

4. Centrifuge the specimens and separate the sera without delay.

5. Determine the amount of dye in the 2 specimens by using the Rosenthal colorimeter supplied by Hynson, Westcott, and Dunning. For this purpose equal amounts of the serum from each specimen of blood are pipetted off into 3 small test tubes of the same diameter as the color tubes of the colorimeter. To one of the tubes add a drop of 10 per cent sodium hydroxide solution. To the others may be added a drop of 5 per cent hydrochloric acid solution if the serum is colored with hemoglobin. Place the tubes in the colorimeter side by side and match by placing the standard tubes behind the tubes without the alkali. The percentage strength of the dye is marked on the standard tube.

The determination may be made also as follows: 1. Prepare a solution of hydrochloric acid by adding 5 cc. of concentrated acid to 95 cc. of distilled water.

2. Dissolve 10 mg. of bromsulfalein (Hyson, Westcott, and Dunning) in 80 cc. of distilled water made alkaline with 0.25 cc. of 10 per cent sodium hydroxide solution and dilute with water to 100 cc. This represents a 100 per cent standard. Prepare

a series of standard tubes of uniform diameter, ranging from 5 to 100 at intervals of 10, diluting the above solution with alkalized water. The standards may be sealed and will keep for several months in the dark. The numbers on the tubes denote amount of dye retained as per cent of amount injected. The dilutions required are as follows:

<i>Tube</i>	<i>Dye</i>	<i>Alk. Water</i>
5	0.25 cc.	4.75 cc.
10	0.50 "	4.50 "
15	0.75 "	4.25 "
20	1.00 "	4.00 "
30	1.50 "	3.50 "
40	2.00 "	3.00 "
50	2.50 "	2.50 "
60	3.00 "	2.00 "
70	3.50 "	1.50 "
80	4.00 "	1.00 "
100	5.00 "	0 "

3. Transfer clear serum to two small test tubes uniform with the standards. To one of these, add 1 or 2 drops of 10 per cent sodium hydroxide solution and to the other 3 drops of 5 per cent hydrochloric acid solution.

4. Compare with the standards in comparator in artificial light, using a white background. Place the acidified tube before the standard and the tube with water in front of the alkalized tube. Read at once to the nearest standard tube. The results are expressed as per cent retention at 30 minutes.

Normally not more than 10 per cent of the dye is retained 30 minutes after its administration and usually only a trace or none at all. A retention of 20 to 40 per cent indicates slight hepatic impairment; 50 to 80 per cent moderately severe and 90 per cent or higher very severe.

### TAKATA-ARA TEST

**Principles.**—This test may give positive results in a wide variety of diseases and especially in parenchymatous diseases of the liver, pulmonary tuberculosis, and nephritis. It is correlated to some extent with changes in the ratio of serum albumin to globulin, but the correlation is not absolute, the mechanism uncertain and the test not very sensitive. Many modifications in the technic have been proposed along with different methods for interpreting the results. It is based on the assumption that mercuric chloride and sodium carbonate form mercuric oxide in the presence of proteins. In pathologic states precipitation of protein (especially globulins) results. May depend upon a change in the constitution of the proteins without necessarily a change in the albumin-globulin ratio.

**Procedure.**—1. Collect 5 cc. of venous blood and separate the serum.

2. In each of a row of 8 test tubes, place 1 cc. of normal saline. Add 1 cc. of serum to the first test tube making serial dilutions of the serum by taking 1 cc. from the first test tube and adding to the second tube and then taking 1 cc. from the second test tube and adding it to the third, and so on to the eighth tube, from which 1 cc.

is discarded. In this way, dilutions of the serum from 1:2 to 1:256 will be obtained. It is also advisable to have a control tube of 1 cc. of normal saline.

3. To each test tube add 0.25 cc. of 10 per cent sodium carbonate and mix well. Then add to each test tube 0.15 cc. of 0.5 per cent bichloride of mercury solution.

4. Read in  $\frac{1}{2}$  hour and again in 24 hours.

5. A positive reaction is said to result when there is a persistent, dense, felt-like flocculent precipitation in at least 3 test tubes, at least one of which should contain the serum in concentrations of 1:32 or higher. As a matter of fact, when the reaction is definitely positive, the precipitation is usually present in more of the tubes. The exact significance of precipitation in less than 3 test tubes is not fully understood but such a result is probably abnormal and should be considered a weakly positive one. A very fine granular precipitation is frequently seen in the higher dilutions and also in the control, but this is without significance. Very coarse flocculi that frequently form in the first 1 or 2 test tubes frequently redissolve on standing and are without significance.

6. As the test was originally done, a mixture of solutions of basic fuchsin and bichloride of mercury was used instead of the latter alone. This has been largely abandoned because the color changes that result have been found to be of no importance.

7. The Takata-Ara test is positive in a very high percentage of patients who have advanced liver disease, particularly cirrhosis.

8. The test can be carried out upon ascitic fluid with the same technic and the results obtained with ascitic fluid have the same significance as those obtained with serum.

9. Under normal conditions the reaction is usually negative.

### HANGER'S CEPHALIN FLOCCULATION TEST

**Principles.**—Recently Hanger<sup>3</sup> proposed a simple test by which disturbances of the liver parenchyma may be detected by noting the capacity of blood serum to flocculate a colloidal suspension of cephalin-cholesterol. The test has the advantage of simplicity and is applicable to jaundiced and non-jaundiced individuals; apparently it has proven of most value in relation to prognosis in cirrhosis of the liver. The reactions expressed in terms of +, ++, +++ and ++++ afford a quantitative estimation of the degree of liver impairment. It combines marked sensitivity with reliability, providing unripened cephalin is used and false weakly positive (+) reactions are ignored and regarded as negative.

**Procedure.**—1. Collect 5 to 10 cc. of blood in a clean, dry test tube after a period of fasting. Separate the serum.

2. Prepare the reagent by dehydrating finely divided sheep brain by 3 extractions with acetone. Dry the tissue and grind into a powder. Extract the powder 3 times with peroxide-free ether. Concentrate the ether extracts *in vacuo*. Add 4 volumes of absolute ethyl alcohol. Decant and collect the precipitate. Dissolve the latter in a minimum amount of ether. Precipitate the cerebrosides by chilling and centrifuging the ether solution. Decant the supernatant ether to which add 4 volumes of absolute ethyl alcohol, chill and filter. Wash the precipitate with absolute alcohol followed by acetone. Dry the precipitate (cephalin) in air and keep in a desiccator in a dark place.

3. Prepare a stock solution by dissolving 0.1 gm. of cephalin and 0.3 gm. of cholesterol in 8 cc. of Squibbs anesthetic ether. Add 1 cc. slowly and with stirring to 35 cc. of freshly distilled water heated to 65 to 70° C. Raise to 100° C. and boil gently until the volume is reduced to 30 cc.; allow to cool (reagent).

4. In a small test tube place 0.2 cc. of patient's serum (which is not over 24 hours old and has been kept in a refrigerator); add 4 cc. of 0.85 per cent sodium chloride solution in distilled water and 1 cc. of the reagent. Shake thoroughly. Keep at room temperature and read after 24 to 48 hours.

5. Positive reactions are indicated by the formation of precipitates which may be recorded as follows: 0 (negative);  $\pm$  (slight flocculation); + (slight flocculation); ++ (moderate flocculation); +++ (marked flocculation); ++++ (complete flocculation with clear supernatant fluid).

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1. Soffer, L. J.: *Medicine*, 14:185, 1935; 2. Quick, A. J.: *Arch. Int. Med.*, 57:544, 1936;
3. Hanger, F. M.: *Jour. Clin. Invest.*, 18:261, 1939; *Am. Jour. Med. Sci.*, 202:48, 1941.



## METHODS FOR THE EXAMINATION OF FECES

**Principles.**—1. An examination of the feces is commonly limited to a search for intestinal parasites or their ova, or for pathogenic bacteria. Much of clinical value can be learned, however, by physical examinations with reference to amount, form and consistency, color, odor and reaction, mucus, etc.; also by chemical examinations for occult blood, bilirubin and urobilinogen, fats and nitrogen, as well as by microscopical examinations for remnants of food, cellular exudates, erythrocytes, parasites, etc.

2. The feces are normally composed of food residues, material secreted through the wall of the intestine and in the bile, leukocytes, desquamated epithelial cells and bacteria. The contents of the ileum are almost liquid with about 400 grams passing into the colon in 24 hours. Much of the fluid is absorbed in the cecum and ascending colon, but small amounts are also absorbed along the transverse, descending and pelvic portions of the colon with the result that the feces evacuated are reduced to about 150 grams.

Food residues, however, constitute a much smaller proportion of the bulk than is usually surmised. Indeed, the fat, protein and carbohydrate of the diet is practically all absorbed, with the result that if the food is free from indigestible material, especially cellulose, the feces are composed almost entirely of intestinal secretions, bacteria, etc. During starvation, for example, feces continue to be formed and their composition does not differ materially from that of feces formed after an ample diet.

3. Due to the comparatively simple dietary of infants, the relatively rapid passage of the intestinal contents, and the absence of intestinal decomposition under ordinary conditions, the results of the analyses of their stools are much easier to interpret than is the case with adults in whom one has to contend with a more complicated dietary and take into account a large number of underlying factors, including the effects of former illnesses, etc.

### MACROSCOPIC EXAMINATION

**Principles.**—In the complete routine examination of feces, attention should be given to the physical characteristics. Much information of clinical value may be derived in relation to amount, form and consistency, color, odor, mucus, concretions, animal parasites, etc. These physical examinations are especially indicated in most patients with suspected gastro-intestinal disease and should rarely be omitted in patients who have diarrhea, constipation, jaundice, anemia, or in infants presenting feeding problems. It is frequently better for physicians to make their own inspection of the feces than to rely alone upon laboratory reports.

**Amount.**—The normal amount of feces for the average adult per day is about 150 to 200 grams. It is increased by a vegetable diet. One or 2 evacuations in 24 hours may be considered normal, yet one every 2 to 4 days is not uncommon in healthy persons. The amount is commonly and characteristically increased in states producing steatorrhea due to an increase of fecal lipids; also in sprue and other conditions accompanied by indigestion of carbohydrates, in which the stools are likely to be large and foamy.

**Form and Consistence.**—The normal stool is soft but formed, being about 1 inch in diameter in adults. Excessively hard feces, sometimes called scybala, are only observed in habitual constipation and indicate atony of the muscular coat

of the colon. In spastic constipation the feces are characterized by numerous hard, ball-like masses. Flattened, ribbon-like stools may result from taking mineral oil but otherwise indicate obstruction of the rectum, generally a carcinoma or a stricture from a healed ulcer, most frequently due to syphilis or lymphopathia venereum. Soft, mushy, or liquid and voluminous stools may follow the administration of cathartics or result from the many causes of diarrhea. In the dysenteries they are invariably small, numerous and largely composed of mucus and blood with small amounts of fecal material.

**Color.**—Normally, in adults, the feces are of a light to dark brown color, chiefly due to urobilin (stercobilin) which is formed from bilirubin by reduction by bacteria into stercobilinogen (urobilinogen) and partly excreted although mostly changed by oxidation into urobilin. On a milk diet, however, the feces are usually of a light yellow color. Likewise in infants, owing partly to their milk diet and partly to the presence of unchanged bilirubin. The ingestion of large amounts of cocoa and chocolate may render them dark gray in color while large amounts of various fruits may give a reddish or black color, spinach and other chlorophyllic vegetables a green, beets a red, rhubarb a yellow, etc.

Drugs may likewise alter the color. For example, the green stools due to biliverdin following the administration of calomel; the dark brown or black stools due to iron and bismuth; the red stools due to neoprontosil; the yellow stools due to santonin or senna; the clay colored stools after a barium meal in connection with x-ray examinations, etc.

Important changes, however, may occur in disease. For example, the "acholic" or clay-colored stools in obstructive jaundice which, however, may be due more to an excess of fecal fat than to a decrease of bile pigments; likewise similar stools, largely consisting of fecal lipids and having a greasy appearance, commonly encountered in achylia pancreatica and tuberculous peritonitis.

Large amounts of blood from hemorrhage in the stomach or upper intestine usually produce tarry black and viscid stools due to digestive changes or dark brown to bright red when the source of bleeding is nearer the rectum. However, when diarrhea exists the color may be red, even if the source of the blood is higher up. Red streaks of blood upon the outside of the feces are due to bleeding from hemorrhoids, fissures, carcinoma or other lesions of the rectum or anus. Blood may be present, however, in amounts too small for recognition by inspection ("occult blood") and require chemical or microscopical tests for its detection.

Green stools are not uncommon, especially in the diarrheas of children, due to biliverdin and sometimes to chromogenic bacteria. They are sometimes seen in healthy infants, alternating with normal yellow stools, and have little significance unless accompanied by symptoms.

**Odor.**—The normal *odor* of feces is due to the presence of aromatic substances, chiefly indole and skatole, derived from the deamination and decarboxylation of tryptophane by putrefactive bacteria in the colon. Therefore, since they are products of decomposition of protein, odor depends largely upon the amount of meat in the diet, being much less on a diet of vegetables or milk.

An increase of odor, however, may be due to other proteins as in the decomposition of large amounts of blood, or of tissues in ulcerated cancers of the sigmoid or rectum. A sour rancid odor due to fatty acids is normal in infants, but may be observed in the diarrheas of older children and adults, along with a highly acid reaction and much

gas formation, largely due to the fermentation of inadequately digested sugars and starches. In severe diarrheas a putrid odor is common while feces emitting a foul stench are suggestive of malignant, syphilitic or other ulcerative lesions of the rectum, gangrenous dysentery, etc.

In addition to indole and skatole, other substances, some of which are toxic, are produced in the colon by the bacterial decomposition of proteins such as histamine, phenol, cresol, ethylamine, etc., including choline formed by the decomposition of lecithin, which, in turn, gives rise to traces of neurine.

**Reaction.**—Normally, the reaction is neutral, slightly acid or slightly alkaline with a pH varying from 6.9 to 7.2. Much depends on the diet as an excess of protein results in alkalinity while an excess of carbohydrates produces acidity. Pathologically, therefore, variations in reaction may result from intestinal indigestion of the respective foodstuffs. Infant's stools are generally acid. The reaction may be determined as follows:

1. Examine as soon as possible after defecation.
2. Thoroughly mix the stool and test with red and blue litmus paper. If the stool is hard, mix with water.
3. Test with Congo red paper.
4. To a watery suspension add a few drops of a 1 per cent alcoholic solution of phenolphthalein (turns pink if alkaline).

**Mucus.**—Normally the feces contain but very small amounts of mucus intimately mixed with the stool. Due care is required against confusing it with mineral oil.

Excessive quantities are easily detected by macroscopic inspection and usually signify irritation or inflammation of the intestine and especially of the colon. When small in amount and intimately mixed with the feces, it is usually derived from the small intestine. Stools composed almost entirely of mucus and streaked with blood are the rule in the acute dysenteries, amebic enteritis and ileocolitis; they may also occur in intussusception.

In so-called "mucous colitis" shreds and ribbons of mucus, sometimes representing complete casts of portions of the colon, are passed, especially after an enema. In the ordinary formed stool the mucus may be unrecognized, unless the feces are well mixed with water, when it may appear as firm, irregularly segmented strands resembling segments of the tape worms, or occur as brown or black jelly-like masses. It is distinguished from catarrhal mucus by the absence of pus cells upon microscopic examination. The disease is probably a secretory neurosis with the name "membranous enteritis" inappropriate.

**Concretions.**—The stools of infants frequently contain whitish curds, due either to fat or casein or a mixture of both. After the ingestion of considerable amounts of olive or other vegetable oils, the feces of adults may show masses of soap and fat which may be mistaken by individuals for gallstones and particularly when given for cholelithiasis. So-called "intestinal sand" is now known to occur particularly in neurotic individuals and composed in most cases of vegetable matter with particular reference to seeds, such as those of berries, bananas, pears, etc.

However, gallbladder stones may occur and should always be looked for in the daily feces of patients over a period of at least 4 days following obscure colicky abdominal pain suggestive of gallstone colic. They are usually readily recognized by

their faceted surfaces; otherwise by chemical examinations for cholesterol and bile pigment. Intestinal concretions (enteroliths) are rare.

### GENERAL MICROSCOPIC EXAMINATIONS

**Principles.**—Microscopical examinations of the feces are usually limited to a search for animal parasites or their ova and cysts. Otherwise, however, additional information of clinical value is frequently to be obtained and especially by an examination of the feces for the numbers and kinds of cells present, although their differentiation and recognition require special skill and experience. Cytological examinations of this kind are particularly valuable in distinguishing between amebic and bacillary dysentery.

**Procedure.**—1. Prepare a thick suspension by rubbing up a portion about the size of a walnut in water (Fig. 120). This gives a uniform mixture more representative than selecting small bits at random.

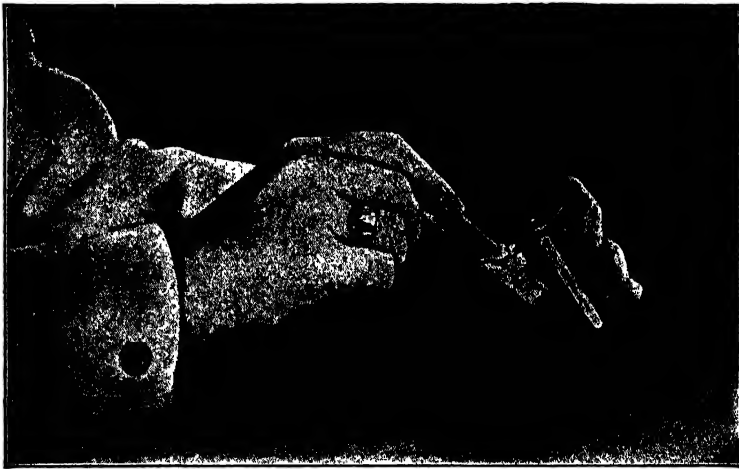


FIG. 120.—MIXING FECES WITH WATER (BENBROOK)

2. Place a drop on a slide and cover with a large coverglass for general examination (No. 1).

3. Place a drop on a slide with 1 or 2 drops of 30 per cent acetic acid for muscle, leukocytes and pus (No. 2).

4. Place a drop on a slide with 1 or 2 drops of Sudan III for fats (No. 3).

5. Place a drop on a slide with 1 or 2 drops of Lugol's solution for starches (No. 4).

6. Examine each microscopically with low and high power with the light well cut down, as is done in the examination of urinary sediments.

7. In this general examination the following may be looked for (Fig. 121):

**Remnants of Food.**—Useful information may be obtained also by examinations for remnants of food as a rough index of the state of digestion. It is advisable, however, for the patient to be on a standard Schmidt diet for this purpose. In this way some idea may be gained relative to the digestion of *starch* (slide No. 4) since the

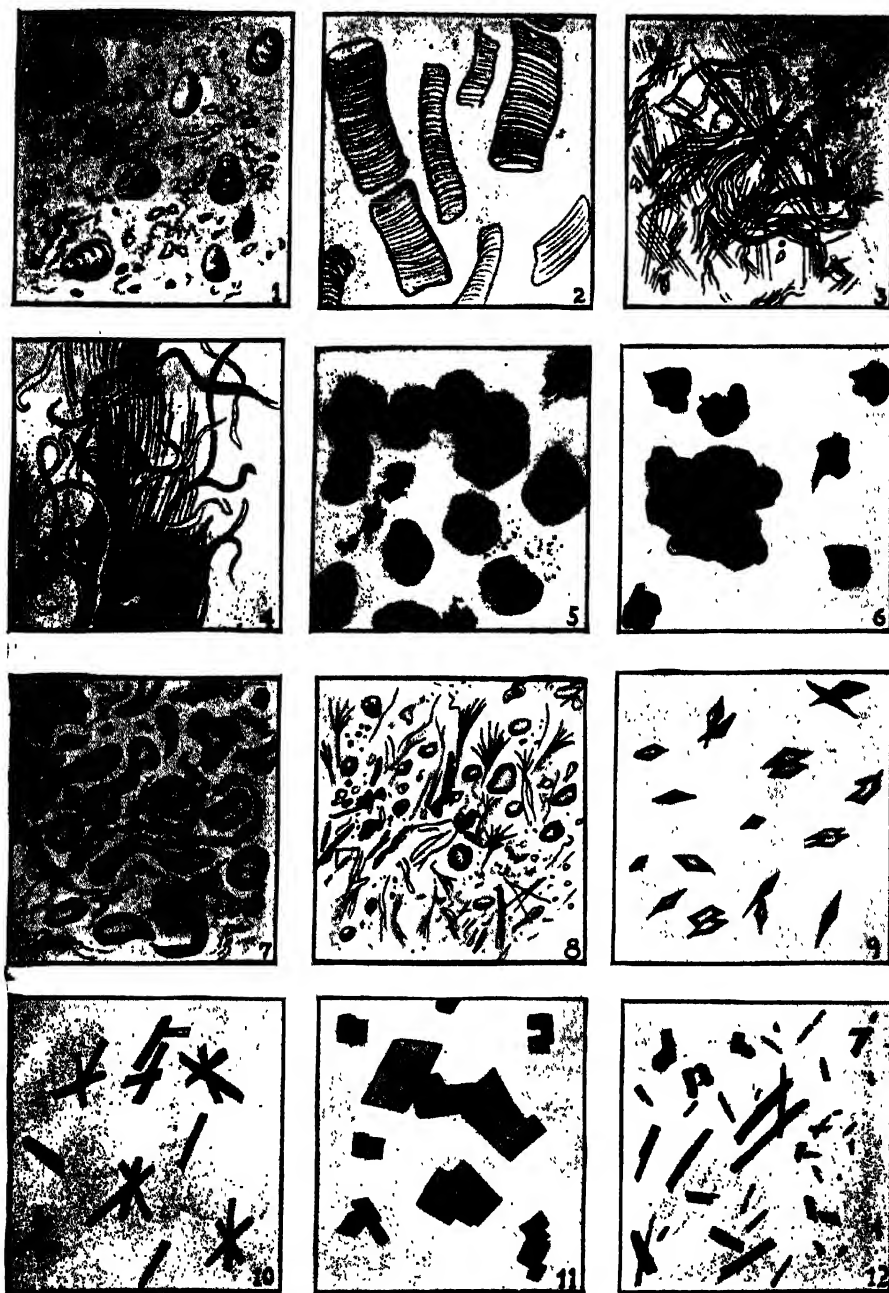


FIG. 121.—MICROSCOPIC EXAMINATION OF FECES

1, Undigested starch granules; 2, partly digested muscle; 3, connective tissue; 4, elastic tissue; 5, neutrophilic leukocytes; 6, epithelial cells; 7, neutral fat; 8, fatty acid and soap crystals; 9, Charcot-Leyden crystals; 10, hematoidin crystals; 11, cholesterol crystals; 12, bismuth oxide crystals.

granules take a blue color if undigested and a red color if partially digested. *Muscle* fibers (slide No. 2) appear as short and transversely striated cylinders with rather squarely broken ends and well preserved nuclei when poorly digested, while presenting rounded ends with no striations at all or but faint ones, with absence of nuclei, when well digested. *Connective tissue*, when but partially digested, is recognizable as yellowish threads with longitudinal striations and *elastic tissue* as well defined branching fibers (slide No. 1).

Some idea may be also obtained relative to the digestion and absorption of *fats* although chemical examinations for total fat are always to be preferred when the possibility of indigestion or faulty absorption are of particular clinical interest. Neutral fats, staining strongly with Sudan III, are normally absent or present in but very small amounts but greatly increased in steatorrhea. Fatty acids may occur as flakes staining orange with Sudan III or as aggregates of needle-like crystals which do not stain at all. Combined fatty acids or soaps (chiefly calcium soap) appear as yellow amorphous flakes or rounded masses resembling ova which do not stain with Sudan III; sometimes they occur as crystals.

**Crystals.**—Additional kinds of crystals may be found also, but few have any clinical significance. These include triple phosphates and the characteristic octahedral crystals of calcium oxalate so likely to be in excess on a vegetable diet. Charcot-Leyden crystals are particularly apt to occur in ulcerative diseases of the colon and especially in amebic dysentery. Yellowish or brown, needle-like or rhombic crystals of hematin may occur after hemorrhages in the gastro-intestinal tract. In this connection it is to be stated, however, that the dark color of feces after the administration of bismuth is largely due to crystals of bismuth suboxide resembling the crystals of hemin. Cholesterin crystals and calcium bilirubinate are found occasionally, especially in cases of cholelithiasis.

**Cytology and Cytodiagnosis.**—Under normal conditions epithelial cells, leukocytes and erythrocytes are not usually present in the feces. *Leukocytes* and *pus* are readily detected (slide No. 2). An excess of *eosinophils* may be found in the mucus discharges of intestinal allergy when smears are stained by the method of Wright, as in differential leukocyte counts of the blood. *Epithelial cells* may show all stages of disintegration and are often unrecognizable. A marked excess of recognizable cells may occur in diseased states.

When bleeding occurs in the small intestine *erythrocytes* can be seldom recognized and the detection of blood depends upon chemical tests. But erythrocytes are usually found in bleeding from the descending colon, rectum, or anus in amebic and bacillary dysenteries (especially the former) as well as in nonspecific ulcerative colitis, ulcerating cancers, hemorrhoids, etc. Furthermore, many investigators have shown that examinations for other cells are of great value in the differential diagnosis between amebic and bacillary dysentery. Naturally this has stimulated inquiry into the relationship of cellular exudates to ulcerative colitis and other types of intestinal disease.

If there are formed stools, preparations should be made of the outside of the fecal mass since it comes into closer contact with the intestinal mucosa. But examinations of the watery stools following a saline cathartic are much more valuable and especially the last portions of stools because they may contain cells from pathological lesions high up in the bowel. But the most valuable material is the terminal mucus evacuated after the last of three saline enemas taken in succession, because gross

fecal material is washed away by the first and second enemas. Precautions should be taken against trauma. The new sigmoid cannula of Bercovitz can be used to aspirate the terminal mucus during sigmoidoscopy.

A small amount of the material may be mixed on a slide with a drop of Loeffler's methylene blue and examined microscopically after being covered with a coverslip; or permanent preparations may be stained with Heidenhain's iron hematoxylin.

With these methods it is possible to recognize and differentiate epithelial cells, polymorphonuclear leukocytes or pus cells, lymphocytes and endothelial or macrophage cells. In amebic dysentery the discharge consists largely of clear mucus sometimes streaked with blood, vegetative motile forms of *Endamoeba histolytica*, and a scanty cellular exudate (Fig. 122). But in bacillary dysentery, the discharge is characterized not only by the absence of amebae but by the presence of heavy cellular exudates composed of pus cells, epithelial cells, lymphocytes and particularly of endothelial macrophages (Fig. 122); the latter must not be mistaken for amebae.

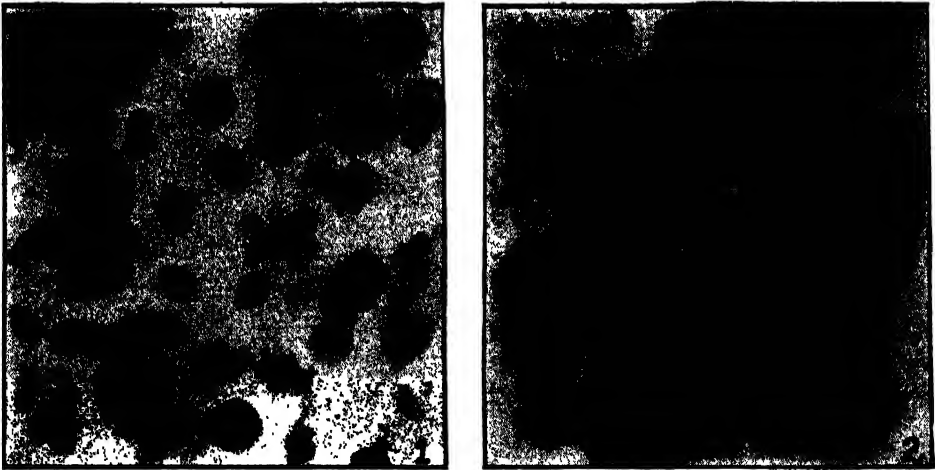


FIG. 122.—FECAL EXUDATES IN DYSENTERY

1, Bacillary dysentery; 2, amebic dysentery.

In some types of diarrhea cellular exudates may not occur but examinations for them are always advisable as aids in the detection of pathological lesions of the intestinal mucosa; when found, they naturally lead to more thorough examinations relative to the location and character of the lesions. Thus heavy cellular exudates have been observed not only in nonspecific chronic ulcerative colitis but likewise in the diarrheas of carcinoma of the colon and sigmoid and in lymphopathiae venereum of women.

### TESTS FOR OCCULT BLOOD

**Principles.**—1. Chemical tests for occult blood depend upon the reaction of the iron of the liberated hemoglobin with the reagent in question.

2. In order to avoid fallacious positive reactions the patient must have been on a meat-free diet for not less than 72 hours before the collection of the specimen. This includes abstinence from fish as well as meat and also the elimination of broths, soups, etc., made from meat stock.

3. As the blood may be unevenly distributed it is important to mix the specimen thoroughly before applying the test.

4. It is well to check a positive reaction by one method by the reaction obtained with another.

**Benzidine Tests.**—1. Prepare a saturated solution of benzidine in glacial acetic acid. Keep in a brown bottle in a dark place, or prepare the reagent freshly as required by adding the amount of crystals picked up on the point of a knife blade to 5 cc. of glacial acetic acid and warm gently to effect solution.

2. Prepare a thin suspension of feces in about 5 cc. of water. Shake with 5 cc. of ether to remove fat. Discard the ether extract. Acidify the residue with acetic acid and again extract with 5 cc. of ether. Evaporate the ether extract to dryness, using a water bath which has been heated to boiling and the flame extinguished. Add 1 cc. of water, stir to dissolve the residue, then add a few drops of benzidine reagent and a few drops of hydrogen peroxide solution. A green to deep blue color indicates a positive reaction.

3. The test may also be conducted by smearing a little of the feces on a slide. Pour over it the reagent made by dissolving a knife-tip of benzidine dissolved in 2 cc. of glacial acetic acid to which is then added 1 to 1.5 cc. of peroxide solution. In a positive reaction, the smear turns blue without any misleading green tints from the reagent.

4. The *Alvarez and Wright modification of the Gregersen and Boas test* is less sensitive but since normal individuals on a meat-free diet do not give positive reactions, it is a valuable check examination although very small amounts of blood may not be detected. The technic is as follows:

(a) Prepare the reagent by mixing 40 gms. of barium peroxide with 5 gms. of pure benzidine. Store in the dark. Just before using, dissolve 0.2 to 0.3 gm. in 5 cc. of 50 per cent acetic acid.

(b) Mix a small amount of feces with a few drops of water. Add a few drops of the reagent.

(c) In the presence of sufficient amounts of blood a blue color develops. Make the readings with a watch according to the following scale:

+ = blue green in 30 to 60 seconds  
++ = vivid blue in 15 seconds  
+++ = deep blue in 3 seconds

**Orthotoluidine Test.**—This is the same as the orthotolidin test of Ruttan and Hardisty except that orthotoluidine is used, which is just as satisfactory and less expensive.

1. Prepare the reagent by dissolving 4 gms. of orthotoluidine in 100 cc. of glacial acetic acid.

2. To 1 cc. of a water suspension of feces add 1 cc. of the reagent and 1 cc. of a 3 per cent solution of hydrogen peroxide.

3. A positive reaction is indicated by the development of a bluish or bluish-green color.

**Phenolphthalein Test.**—1. Prepare the reagent by dissolving 2 gms. of phenolphthalein and 25 gms. of potassium hydroxide in 100 cc. of distilled water; add 1 gm. of powdered zinc and heat gently until decolorized. The reagent is stable.



2. Make a thin suspension of feces in about 5 cc. of distilled water and heat to boiling to inactivate the oxidizing enzymes.
3. Allow to cool and to 1 cc. of reagent add 2 cc. of the heated suspension and then add a few drops of hydrogen peroxide.
4. A pink to reddish color indicates a positive reaction.

### TESTS FOR UROBILIN

**Principles.**—1. The word “urobilin” is here used as a synonym for hydrobilirubin and includes its mother substances, bilirubin and the chromogen, urobilinogen.

2. Owing to constipation and other factors, the amount is subject to variation, although the total daily output is fairly uniform.

3. Since the mother substance, bilirubin, is a product of hemoglobin, an estimation of urobilin in the feces is an approximate index of blood destruction and has had a useful application in differentiating between pernicious anemia and the secondary anemias due to hemorrhage.

4. Urobilin is absent or greatly reduced in obstructive jaundice and its return to the feces is often the first sign of relief.

**Schmidt's Qualitative Test.**—This test depends upon the formation of hydrobilirubin-mercury with the production of red color.

1. Rub up a small amount of feces in a mortar with a saturated watery solution of mercuric chloride.

2. Transfer to a shallow white dish and let stand for 6 to 24 hours.

3. The presence of hydrobilirubin or urobilin is indicated by a deep red color being imparted to the particles of feces containing the pigment.

If unaltered bilirubin is present, a green color is produced through its oxidation to biliverdin.

**Quantitative Test of Wilbur and Addis.**—This method depends upon extraction of hydrobilirubin and its quantitative estimation by spectroscopic examination.

1. Collect all the feces for 24 hours, keeping them in darkness.

2. Grind the whole quantity with water to a homogeneous paste.

3. Dilute to 1000 cc. with tap water (or to 500 to 2000 cc. if the amount of feces is unusually small or large).

4. Measure off 25 cc. and add to this 75 cc. acid alcohol (alcohol 64 cc., concentrated hydrochloric acid 1 cc., water 32 cc.).

5. Place in a mechanical shaker for  $\frac{1}{2}$  hour. Constant shaking by hand for a similar period will suffice.

6. Add 100 cc. of saturated alcoholic solution of zinc acetate, and filter.

7. To 20 cc. of the filtrate add 2 cc. of Ehrlich's reagent (paradimethylaminobenzaldehyde, 20 grams; concentrated hydrochloric acid, 150 cc.; water 150 cc.).

8. Keep in darkness until next day (or at least for 6 hours) and examine spectroscopically. In the presence of both urobilinogen and urobilin, the absorption bands indicated in Figure 123, A and B, will be seen.

9. Dilute with 60 per cent alcohol, adding a few cc. at a time, until first one and then the other band has entirely disappeared when the slit of the spectroscope is wide open, but still remains visible when the slit is partly closed. The end-point is fairly definite after one has established the standard upon a series of normal stools. It

is perhaps best to use an unvarying width of slit and to dilute until the bands have just disappeared with this opening. One may establish uniform conditions as to the thickness of the layer of fluid, the kind and strength of the light, and the distance from the light, and then adopt a width of slit which gives an average of about 6000 dilutions in a series of normals. When using the "pocket" type of spectroscope, place the fluid in a standard serologic tube about

12 millimeters in diameters, and employ a 60-watt frosted Mazda lamp, placed about 6 inches from the spectroscope, which is mounted upon a temporary stand to insure steadiness. The eyes are protected from the light by a cardboard screen.

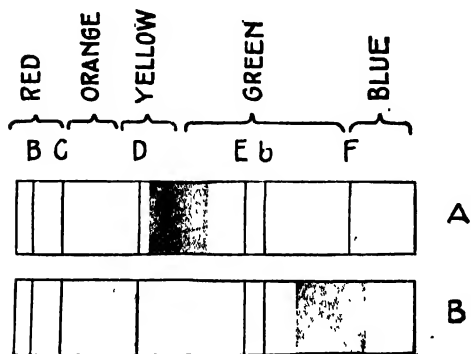


FIG. 123.—ABSORPTION SPECTRA

A, urobilinogen in acid solution with Ehrlich's reagent; B, urobilin in acid solution with zinc acetate. (From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

10. Calculate separately the number of dilutions necessary to cause disappearance of each of the absorption bands and add the two together. The calculation is based, not upon the 20 cc. of filtrate used, *but upon the 2.5 cc. of fecal suspension represented by the filtrate*. The dilution value for the 24-hour stool (1000 cc. of fecal suspension) is then found by multiplying this figure by 400. When the fecal suspension is made up to 500 or 2000 cc. the multiplier would, of course, be 200 or 800. This

final result indicates the number of dilutions which would be necessary if all the urobilin and urobilinogen of the 24-hour stool were concentrated in the 2.5 cc. of fecal suspension examined.

Example: Suppose that in step 9 the urobilinogen band disappeared when the 20 cc. of filtrate had been diluted to 25 cc., and the urobilin band when the volume reached 30 cc., then the dilution values for the 2.5 cc. of feces would be 10 and 12 respectively and the combined value  $10 + 12 = 22$ . The total dilution value of the 24-hour stool would then be  $22 \times 400 = 8800$ .

11. The normal dilution value is about 6,000; 9,000 is the upper limit of normal.

## HUPPERT'S TEST FOR BILIRUBIN

**Principles.**—This test is based upon the precipitation of bilirubin by milk of lime followed by its solution in alcohol to which it imparts a green color. Normally, bilirubin is never present in the feces of adults. But in enteritis accompanied by diarrhea it may be present and traces may occur also in obstructive jaundice.

**Procedure.**—1. Prepare milk of lime by mixing 1.5 gms. calcium oxide, U.S.P., with 1000 cc. of distilled water.

2. Prepare a sodium nitrite reagent by dissolving 0.1 gm. in 10 cc. of distilled water.

3. Mix fresh feces with enough distilled water to form a semifluid mass. Strain through cheesecloth.

4. Add an equal volume of milk of lime, to the semifluid feces, shake thoroughly and filter through paper. Wash the precipitate with water.
5. Transfer both the precipitate and the filter paper to a small beaker. Add 5 cc. of 95 per cent alcohol, acidified slightly with sulfuric acid, and heat to boiling on a water bath. Add 1 drop of the sodium nitrite reagent.
6. The presence of bilirubin is shown by the alcohol assuming a green color.

### TEST FOR BILE ACIDS

1. Extract a small amount of feces with alcohol, and filter.
2. Evaporate the filtrate in a dish over a water bath.
3. Dissolve the residue in water made slightly alkaline with potassium hydroxide solution.
4. Add 0.3 cc. of a 5 per cent solution of sucrose.
5. Transfer to a test tube and carefully run down the sides about 3 cc. of concentrated sulphuric acid. Cool the tube in running water so that the temperature does not go above 70° C.

A red ring at the point of contact is a positive reaction. Upon slight agitation the contents of the tube assume a reddish color.

### TESTS FOR PANCREATIC ENZYMES

**Principles.**—Two of the pancreatic ferments—amylase and trypsin—are normally present in feces. Lipase cannot usually be detected.

In pancreatic disease and obstruction of the pancreatic duct these ferments may be diminished or absent. Quantitative tests, therefore, may be of diagnostic value, especially in conjunction with an estimation of amylase in the urine.

Tests for both ferments should be done although that for amylase is the more useful of the two, since the action of trypsin may be simulated by erepsin and proteolytic bacteria.

**Securing Specimen of Feces.**—1. Upon the evening before the test, limit the patient to a light supper and give a high enema at bedtime.

2. At 7 next morning give 750 cc. (25 ounces) of milk.
3. At 7:30 give  $\frac{1}{2}$  ounce Epsom salts; repeat at 8.
4. At 8:30 give a glass of water containing  $\frac{1}{4}$  teaspoonful of sodium bicarbonate.
5. Save all the feces passed up to 2 P.M. in a vessel containing 2 ounces of toluol. Keep in a cool place. If less than 400 cc. are obtained, give an enema of 1 pint of water.
6. Dilute the whole volume of feces to 3000 cc. with normal salt solution, mix well, and centrifugalize a portion for 5 minutes. Use the supernatant fluid for the following tests:

**Test for Amylase.**—1. Prepare a 1 per cent solution of soluble starch as follows: To 100 cc. of cold distilled water, add 1 gm. soluble starch (Kahlbaum's recommended) and heat gently with constant stirring until clear.

2. Place 2 cc. of this solution in each of 12 test tubes.
3. To these tubes add the supernatant fluid from the centrifugalized feces as follows:

No. 1:1.8 cc.	No. 7:0.6 cc.
No. 2:1.6 cc.	No. 8:0.4 cc.
No. 3:1.4 cc.	No. 9:0.2 cc.
No. 4:1.2 cc.	No. 10:0.1 cc.
No. 5:1.0 cc.	No. 11:0.05 cc.
No. 6:0.8 cc.	No. 12:none (control)

Bring the quantity in each tube up to 4 cc. with normal salt solution.

4. Place the tubes in an incubator or water bath at about 38° C. for ½ hour.

5. Fill all tubes with tap water and add a drop of weak iodine solution to each. Gram's iodine solution will suffice.

6. If amylase is present, the series of tubes will vary from yellow through reddish-purple to pure blue, depending upon complete or partial digestion of the starch. The tube before the one in which the first definite trace of blue appears is taken as the measure of digestion. In normal individuals it is usually found to be either the ninth or tenth tube, corresponding to 30,000 and 60,000 units respectively.

**Gross Test for Trypsin.**—1. Prepare a 1:1000 solution of casein by dissolving 0.1 gm. of casein (C.P.) and 0.1 gm. of sodium bicarbonate in 100 cc. of distilled water. Boil for 1 minute, stirring constantly, and cool.

2. Place 5 cc. of the casein solution in each of 12 test tubes and add to these tubes the same amounts of the fecal suspension, previously filtered, as were used for the amylase test.

3. Place the tubes in the incubator or a water bath at 38° C. for 1 hour.

4. Test for digestion of casein by adding a few drops of 3 per cent acetic acid to each tube and mixing gently. Digestion is complete in those tubes in which no white precipitate forms and the tube before the one in which the first definite precipitate appears is taken as the measure of proteolytic activity (nearly always the fourth tube). The end-point is less definite than in the test for amylase.

## TESTS FOR FATS

**Principles.**—1. Even on a fat-free diet there is a daily excretion of about 2 grams of fat, the composition of which is very similar to that of the blood lipids. Indeed, it has been shown that the amount of fat in the feces and its composition are to a large extent independent of that ingested with foods and, consequently, cannot be regarded as representing entirely a residue from the fat of the diet. Under the circumstances it is now generally believed that at least a portion of the fecal lipids is derived from the blood by excretion into the small intestine.

2. On an unregulated diet considerable variation in the amount of fat in the feces of normal individuals is naturally to be expected and even in the same person from day to day. Thus, total fat in dry feces has been found to vary from 7.3 to 27.6 per cent per day (average 17.5 per cent). Of this, neutral fat averages 7.3 per cent; free fatty acids 5.6 per cent, and combined fatty acids (soaps) about 4.6 per cent. Normally, on the Schmidt diet over 94 per cent of the fat is absorbed so that 6 per cent or less of the dried feces is composed of fat. Under the circumstances a total fat amounting to more than 25 per cent of the dried feces is probably abnormal; likewise, a neutral fat exceeding 11 per cent (evidence of deficient fat splitting) and more than 16 per cent of fatty acids combined as soap (evidence of deficient fat absorption).

3. An excess of fat in the feces is designated as *steatorrhea*. This may occur in enteritis involving the small intestine in which large amounts of undigested and unabsorbed fat are rushed through into the colon with an increase of total fat due to an increase of neutral fat and fatty acids, both free and combined (soaps).

**Qualitative Tests.**—Neutral fats, fatty acids and soaps are best detected and differentiated by quantitative chemical methods. Otherwise, useful data is sometimes obtained by rubbing up a small portion of feces on a slide with 36 per cent solution acetic acid, applying a coverglass and heating over a flame until the preparation shows bubbles (No. 1). Duplicate slides are prepared in the same manner, to one of which Sudan III solution is allowed to flow under the coverglass (No. 2) and to the second a solution of Scharlach R (No. 3). Both stains are saturated solutions in equal parts of 70 per cent alcohol and acetone. The slides are examined microscopically. Additional tests may be conducted and the results interpreted as shown in Table 12.

TABLE 12

Test	Neutral Fats	Fatty Acids	Soaps
Microscopic (No. 1)	Round or irregular globules; highly refractile or minute needles	Sheaves of large needles or short delicate curved needles which occur in such thick masses that the shape of the individual crystals can seldom be made out	Needles arranged in clusters or fans or in short plump crystals or scales. In amorphous form as gnarled bodies everted like the pinna of an ear. Soap crystals are comparatively coarse, as a rule (thick short needles or flakes), but may be indistinguishable from those of fatty acids
Heat solubility . . . . .	Melted	Melted	Not melted
Ether solubility . . . . .	Dissolved	Dissolved	Not dissolved
Scharlach R * (No. 3)	Stained	Crystals not stained. Globules stained	Not stained
Sudan III * (No. 2) . .	Stained	Light orange crystals not stained	
Water solubility . . . . .	0	0	Sodium and potassium soaps dissolved. Calcium and magnesium soaps not dissolved

\* Scharlach R and Sudan III solutions are saturated solutions in equal parts 70 per cent alcohol and acetone.

**Saxon's Quantitative Test.**—The soaps are converted into free fatty acids by means of hydrochloric acid, and the material is then extracted by shaking with ether. The ether removes the neutral fat, the fatty acids present as such, the fatty acids from the soaps, and the cholesterol. The ether is removed, the crude fat purified by means

of petroleum ether, and the weight of the total fat obtained. The fat is then dissolved in benzene and titrated with N/10 sodium alcoholate solution, using phenolphthalein as an indicator. The fatty acid is calculated, from the titration, as stearic acid.

1. Place about 5 grams (accurately weighed) of thoroughly mixed feces in a 100 cc. glass-stoppered graduated cylinder, care being taken not to smear the neck of the cylinder. This procedure is best carried out by weighing a small evaporating dish of feces along with a small spatula before and after transfer.

2. Add 20 cc. of distilled water, 1 to 2.5 cc. of concentrated hydrochloric acid (depending upon the amount of the sample) and sufficient water to make a total bulk of 30 cc. Add exactly 20 cc. of ether, stopper, and shake vigorously for five minutes. Allow to stand a few seconds, remove the stopper, add exactly 20 cc. of 95 per cent alcohol, and again shake for five minutes.

3. Stand the cylinder aside. The ether, containing practically all of the fat, will come to the top as a colored transparent layer. Blow the ether off into a 200 cc. Erlenmeyer flask, making use of the syphon principle as employed in the wash bottle, the submerged end of the delivery tube being bent upward. The thin layer of ether which remains is diluted with 5 cc. of ether, the tube slightly agitated, and the ether blown off. This is done in all five times, care being taken each time to wash down the sides of the cylinder. The stopper should also be washed.

4. Twenty cc. of ether are again added and the cylinder shaken for 5 minutes and set aside. When the ether has nearly stratified, blow it off and wash as before. During the second washing stratification will complete itself.

5. Using a hot water bath, distill off the ether until no trace of ether remains. This is assured by finally bringing the water bath to a boil for a few minutes. To the residue add 30 cc. of low-boiling petroleum ether. Stopper with a cork and allow to stand overnight. Petroleum ether for this work should boil below 60° C. It should be tested for a residue on evaporation and must be redistilled if such is present.

6. Filter the petroleum ether solution of the fat, catch the filtrate and petroleum ether washings in a tall 100 cc. beaker which has been previously heated in an oven at 100° C., transferred to a desiccator and weighed. Evaporate off the solvent on a clean surfaced electric hot plate (being careful not to overheat near the end). Dry in an oven at 100° C., cool in a desiccator for 20 minutes and weigh. The difference in the two beaker weighings represents total fat in 5 grams of feces.

7. After weighing, dissolve the contents of the beaker in 50 cc. of benzol, heat almost to the boiling point, add 2 drops of a 0.5 per cent solution of phenolphthalein, and titrate with an N/10 solution of sodium alcoholate.

8. The weight of fatty acids (in terms of milligrams of stearic acid) is obtained by multiplying the number of cc. of N/10 sodium alcoholate solution by the factor 28.4.

9. The difference between the weight of the total fat and the weight of the fatty acids is the weight of the neutral fat.

NOTE.—In order to facilitate separation of the ether and water it may be desirable to put the cylinder in a centrifuge.

## METHODS FOR CONDUCTING PANCREAS FUNCTION TESTS

**Principles.**—1. The chief and apparently sole functions of the pancreas are in connection with digestion and metabolism through its internal and external secretions. The internal secretion, insulin, produced by the islands of Langerhans and absorbed directly into the blood, plays an important part in the metabolism of carbohydrates. The external secretion provides trypsin, rennin, traces of erepsin, amylase, small amounts of maltase and lipase for the digestion of proteins, starches and fats respectively. It is strongly alkaline in reaction and contains a small amount of coagulable protein as well as inorganic constituents with special reference to sodium carbonate and chlorides.

2. Examination for trypsin, amylase and lipase in the duodenal contents are usually helpful in the detection of pancreatic disease. Simple qualitative tests have been described on pages 218 to 220, but in examinations for these enzymes in relation to the functional activity of the pancreas, it is advisable to collect duodenal contents and conduct the tests by special methods described herewith.

3. In suspected disease of the pancreas examinations of the feces for excessive amounts of undigested meat fibers are likewise of clinical value. The same is true of examinations of the feces for total nitrogen since the presence of 25 per cent or more of the nitrogen ingested in foods (*azotorrhea*) is of frequent occurrence in chronic pancreatitis. But of greater clinical value is an examination of the feces for the total lipids (pages 250 to 251), since an increase of them (*stecatorrhea*) is usual in achylia pancreatica due to chronic pancreatitis, extensive carcinoma of the pancreas, calculus obstruction of the ducts, etc. However, a chemical differentiation of the lipids into their various kinds (neutral fat, soap fat and fatty acids) is of limited clinical value.

4. Furthermore, examinations for insulin deficiency are frequently helpful since glycosuria, hyperglycemia and diminished sugar tolerance may be found in about 35 per cent of cases with acute pancreatic lesions due to involvement of the islands of Langerhans, although but seldom observed in chronic pancreatic disease. Since the starch-splitting enzyme of the blood (amylase or diastase) appears to be partly of pancreatic origin, the quantitative determination of this enzyme in the blood has proven of value and particularly in acute pancreatitis. Indeed, a normal blood amylase is stated to be important evidence against the existence of pancreatitis as a cause of acute abdominal symptoms, since an increase has not been found in conditions other than pancreatic disease.

5. A quantitative determination of the amylase (diastase) in the urine is also of value in the recognition of acute pancreatitis. The cause of increased blood and urine amylase is an open question although it is thought that the inflammatory process may obstruct the finer pancreatic ducts which forces the enzyme into the blood.

6. In this connection it may be also stated that a large proportion of patients with acute and subacute pancreatitis show in the urine some substance (believed to be in the nature of a glyco-nucleoprotein) which, upon hydrolysis, yields a pentose capable of forming an osazone (*Cambridge reaction*). However, careful studies reported by many investigators have not substantiated the clinical value of the test, although a strongly positive reaction may aid in establishing the diagnosis of acute or subacute degenerative lesions of the pancreas in the presence of clinical signs and symptoms.

### LUEDERS AND HOLLANDER TESTS FOR ENZYMES IN THE DUODENAL CONTENTS

**Principles.**—These tests of the duodenal contents for trypsin, lipase and amylase are based upon the assumption that a deficiency is indicative of pancreatic disease. If trypsin and amylase are present in normal amounts it is not usually necessary to examine for lipase.

**Collection.**—Follow the regular Lyon technic for duodenal drainage (pages 210 to 215) except for the departures indicated:

1. Do not permit the patient to swallow saliva during the procedure.
2. Test the removed gastric contents for free HCl. If it is absent, introduce 100 cc. of HCl (0.5 per cent) through the tube with the patient recumbent. Retain for 5 minutes in an effort to destroy salivary amylase. Remove the acid by gravity flow while the patient gradually swallows the tube to the 70 cm. mark (20 minutes). Gravity return is the safe and correct method for collection of duodenal juice.
3. When the tube reaches the designated mark, the bile-tinged duodenal residue should begin to flow. Make the collection in a test-tube surrounded by ice. Discard if free HCl is present (test repeatedly). Finally collect 10 cc. of clear fluid. If this cannot be obtained, pass 50 cc. of hot water through the tube and induce immediate return. Mark the fluid subsequently collected as sample "A" and place on ice until analyzed.
4. Stimulate flow of bile with 37.5 cc. of 33 per cent  $MgSO_4$ . Clamp the tube for 5 minutes. When the return flow becomes a clear dark brown, collect 10 cc. as before. Mark this tube "B". Collect sample "C" in the same manner. If the fluid is cloudy, turn the patient on his back.

Discard all samples which show free HCl or a total acidity above 10. Estimate the enzymes at once or store on ice.

**Test for Trypsin.**—Trypsin is estimated as to concentration and is expressed in minutes required for 0.5 cc. of duodenal fluid to neutralize 1 cc. of 0.1 N NaOH, when both are added to a neutral emulsion of 5 per cent gelatin (rendered pink with phenolphthalein. In quantitative determination of reversible reactions (as typified by proteolytic and lipolytic activity of duodenal juice) it is more accurate to measure the time taken to effect a definite change than the amount of change in a definite time (Hollander). A normal tryptic activity will, within 5 minutes, produce amino acids sufficient to neutralize the mixture with consequent loss of pink coloration.

1. Add 25 cc. of neutral 5 per cent emulsion of gelatin to each of 4 large test-tubes marked "A", "B", "C", and "X" which is the control. To "X" add 1 cc. of boiled duodenal fluid of either "A" or "B" fraction, heat having destroyed the enzyme.
2. Place the tubes in a water-bath preferably in a copper rack. Maintain the temperature at 37° to 40° C., warming if necessary with a microburner.
3. Add the exact amount of 0.1 N NaOH needed to equalize the low acidity of 0.5 cc. of the duodenal fluid in the 3 fractions. Transfer the 0.5 cc. of each fluid to the proper tube, noting the exact time for each addition. Remove each tube, shake well and return to the bath.
4. Add 0.5 cc. of 0.1 N NaOH to tubes "A", "B", and "C". Shake and return to the bath. When the dark pink changes to a light pink, add the final 0.5 cc. of alkali to each tube and shake.
5. Note the exact time when the pink color disappears from the three solutions,



or when it matches the solution in tube "X". Express the activity in minutes and fractions thereof. The activity is inversely proportional to the concentration.

**Tests for Lipase.**—The activity is expressed in terms of the time required for 0.1 cc. of duodenal fluid to neutralize 1 cc. of 0.1 N NaOH, when both are added to a neutral emulsion of 20 per cent olive oil rendered pink with phenolphthalein (1 per cent). A normal concentration of lipase will, within 5 minutes, produce fatty acids sufficient to neutralize the alkali with disappearance of the pink color. If neutralization takes longer than 5 minutes, use 0.5 cc. of duodenal fluid instead of 0.1 cc. and multiply the time by 5.

1. Add 25 cc. of neutral emulsion of 20 per cent olive oil to each of 4 tubes marked as in the foregoing. Prepare a similar control tube and heat in the same manner.

2. Add the exact amount of 0.1 N NaOH to equalize the low acidity of the duodenal fluid. (Estimate the exact amount by testing the acidity of 1 cc. of the sample; add to the solution  $\frac{1}{2}$  the reading if 0.5 cc. of duodenal fluid is analyzed; make no adjustment for the 0.1 cc. of fluid.)

3. Add 0.1 cc. of duodenal samples to the proper tubes, shake and return to the bath. Note the exact time that each is added to the substrate.

4. Wait exactly 3 minutes. (This permits the enzyme to act in a neutral or weakly acid mixture; the large amount of alkali compared to the quantity of juice may inhibit the lipase.) The pink color may disappear.

5. Add 0.5 cc. of 0.1 N sodium hydroxide solution to tubes "A", "B", and "C". A deep pink color may appear. When the mixture loses its color completely, add the final 0.5 cc. of alkali. Note the exact time when the color in each of the 3 tubes matches tube "X". If upon addition of the second 0.5 cc. of alkali, no pink color recurs, the ferment concentration is less than "3 minutes". Repeat the test, adding the 2 half-portions of alkali within the first minute of digestion.

**Test for Amylase.**—Amylase is determined quantitatively by estimating the maltose formed by the action of 1 cc. of duodenal fluid on 20 cc. of a 5 per cent solution of potato starch made neutral or faintly pink with phenolphthalein (0.2 per cent, alcoholic). The end-point is yellow-white to light gray. Beyond the end-point, a deepening pink color appears. Since the substrate contains 1 gm. of starch, the total amount of maltose formed in 1 hour will be a fraction of a gram. According to Bergeim, the actual hydrolysis seldom exceeds 80 per cent of the theoretical 1.056 gm. of maltose from 1 gm. of starch. Willstätter considers 75 per cent the limit of saccharification. The normal findings are presented in Table 13.

1. Add 20 cc. of 5 per cent starch solution to each of 4 tubes as in the foregoing tests. Add to each tube 1 drop of 0.2 per cent phenolphthalein, then dropwise normal sodium hydroxide solution until a pale pink color persists on shaking (usually 1 to 2 drops).

2. Just neutralize the alkali in each tube with 0.1 N  $H_2SO_4$  from a burette. (The pH for maximum amylolytic activity is 6.8; the addition of the mineral acid and the weakly acidic duodenal fluid provide a reaction close to pH 7.) Immerse the tubes in warm water until the contents are heated to 37° C. (10 minutes).

3. Add 1 cc. of each duodenal fluid to the proper tube (1 cc. of boiled juice to tube "X"). Incubate for 1 hour at 37° to 40° C., shaking every 15 minutes. On re-

TABLE 13.—CALCULATION OF TOTAL MALTOSE

Normal Hydrolysis		Poor Hydrolysis	
Burette reading (ml.)	Maltose (gm. and per cent)	Burette reading (ml.)	Maltose (gm. and per cent)
0.3 . . . . .	0.99*	1.1 . . . . .	0.27
0.4 . . . . .	0.74	1.2 . . . . .	0.25
0.5 . . . . .	0.60	1.3 . . . . .	0.23
0.6 . . . . .	0.50	1.4 . . . . .	0.21
0.7 . . . . .	0.42	1.5 . . . . .	0.20
0.8 . . . . .	0.37	1.6 . . . . .	0.19
0.9 . . . . .	0.33	1.7 . . . . .	0.17
1.0 . . . . .	0.30	1.8 . . . . .	0.16

\* The conversion of 1 gm. of starch to 0.99 gm. of maltose is exceptional. Readings lower than 0.3 ml. would suggest the presence of salivary amylase or intestinal amylase in sufficient quantities to vitiate the test.

moval from the bath or incubator, stop digestion with approximately 300 mg. of  $\text{Na}_2\text{CO}_3$ .

4. Transfer to a large pyrex test-tube or a 25 cc. Erlenmeyer flask 5 cc. of Benedict's quantitative reagent, a pinch of powdered talc, and 1 to 2 gm. of  $\text{Na}_2\text{CO}_3$ . Heat to boiling, then add the digestion mixture from a 2 cc. graduated pipette as in urinalysis. Boil *gently* until the last blue color disappears and changes to yellow or slate-gray. If pink is encountered, the end-point has been overstepped.

5. **Calculation:** 0.0149 mg. of maltose reduced 5 cc. of Benedict's reagent.  

$$\frac{0.0149}{\text{cc.}} \times 20 = \text{grams of maltose formed from 1 gm. of starch. (Consult Table 13.)}$$

## FECES TESTS

**Principles.**—These tests for pancreatic function are based upon examination of the feces for the digestion of muscle fibers (especially of their nuclei) and chemical examinations for fats and nitrogen.

**Schmidt Nuclei Test.**—1. This test consists in the administration with a meal of a 0.5 gm. cube of beef or, better, of thymus, tied in a little gauze bag. The meat must previously have been hardened in alcohol and well washed in water.

2. When the bag appears in the feces it is opened and its contents examined microscopically. If the nuclei are for the most part undigested, pancreatic insufficiency may be assumed, since it is probable that nuclei can be digested only by the trypsin of the pancreatic juice. Normally the nuclei are digested, provided the time of passage through the gastro-intestinal tract is not less than 8 or 10 hours. However, if the time exceeds 30 hours, nuclei may be partially digested in the complete absence of pancreatic trypsin.

**Schmidt Standard Diet Test.**—This test may be employed not only for the determination of the digestion of protein (muscle and nuclei) but for starch and fats as well. The determination of fat by chemical analysis and of muscle for digestion by microscopical examinations are of most value.

1. The diet is given for 3 days with carmine (0.3 gm. in a capsule) at the beginning to identify the feces. The morning after the diet is ended charcoal (15 gm.) is

given with a breakfast consisting entirely of milk. After the beginning of the test diet, the first stool colored red is saved and examined as are all succeeding ones, until the appearance of the first stool colored with charcoal, which is rejected. The diet is as follows:

Mornings: 0.5 liter of milk with 50 mm. zweibach.

Forenoons: Oatmeal gruel (40 gm. rolled oats, 10 gm. butter, 200 cc., milk, 300 cc. water and 1 egg, and salted to taste—strain).

Noons: 125 gm. finely chopped roast beef lightly broiled *so that the interior remains uncooked*. In addition, 250 cc. potato purée (potato 190 gm., milk 100 cc., butter 10 gm., salted to taste).

Afternoons: As mornings.

Evenings: As forenoons.

2. Examine all of the stools showing the presence of carmine for digestion of muscle, starches and fats as described on pages 248 to 251.

**Beazell, Schmidt and Ivy Diet Test.**—1. In this test the patient is placed on a standard balanced diet supplying 64 gm. of protein (10.3 gm. of nitrogen) and 112 gm. of fat daily for a period of 6 days. Carmine (5 grains) is given with the first meal, again with breakfast on the fourth day and finally with the first meal after the standard diet has been discontinued. Starting with breakfast on the fourth day, the patient is given with each meal 8.0 gm. of potent pancreatin in the form of enteric coated tablets.

2. At the time of the appearance of the first dose of carmine in the stool, collection of the total fecal output is started and continued until the last dose of carmine makes its appearance. The carmine given on the fourth day serves to separate the control period from the enzyme period.

3. Immediately after passage the feces are transferred to an air tight bottle containing 5 cc. of toluene and stored in a refrigerator.

4. Each day the total fecal output for the preceding 24 hours is weighed, thoroughly mixed and sampled for chemical determination. The samples for each period (control and enzyme) are pooled and the determinations are carried out on the composite samples.

5. The samples for the nitrogen determination are suspended in sulfuric acid as recommended by Peters and Van Slyke; samples for the determination of fat are preserved in alcohol, which is then used for the initial lipid extraction.

6. Nitrogen is determined by the Kjeldahl method and fat by a modification of Saxson's method. All determinations are made in duplicate. No information of definite diagnostic value is obtained by determining the ratio of undigested fat (neutral fat) to digested fat (soaps and fatty acids) as it has been shown that even in the absence of pancreatic digestion the ratio may be normal.

7. Normally the average daily fecal nitrogen varies from 1.5 to 2.3 gm. and the lipids from 7 to 10 gm. In a series of four cases of achylia pancreatica Beazell and his colleagues found the nitrogen to vary from 4.15 to 13.30 gm. and the lipids from 44.5 to 94.0 gm. during the control period before the administration of pancreatin. During the period in which the enzyme was given, the nitrogen varied from 1.57 to 3.7 gm. and the lipids from 21.1 to 32.7 gm. In other words, this method reveals the excess

of both nitrogen and fats in the feces in achylia pancreatica with increased absorption of both when enzyme therapy is instituted.

### **SOMOLGI PLASMA TEST FOR AMYLASE**

In this test the concentration of amylase in the plasma is determined by its action on a starch paste substrat with subsequent determination of the starch cleavage products on the basis of copper-reduction activity. The technic is given on pages 851 to 852. For its conduct collect 10 cc. of blood from a vein in oxalate after a period of fasting as for the blood glucose test. Separate the plasma by centrifuging.

### **CHERRY AND CRANDALL SERUM TEST FOR LIPASE**

This test is based upon the specificity of pancreatic lipase and its occurrence in the blood in pancreatic disease. The substrat used is an emulsion of olive oil. The concentration of the lipase is indicated by the amount of fatty acid liberated, and is reported in terms of cubic centimeters of twentieth-normal solution of sodium hydroxide required for neutralization. The test is described on pages 850 to 851. For its conduct collect 10 cc. of blood in a chemically clean test tube after a period of fasting and separate the serum.

### **URINE TESTS FOR AMYLASE**

A small amount of amylase occurs normally in the urine. In acute pancreatitis and in obstruction of the common bile duct there may be a marked increase. In other pathologic conditions of the pancreas or liver, it is usually within normal. Tests are based upon the digestion of starch as detected by an iodine indicator and are described on pages 153 to 154.

## METHODS FOR THE EXAMINATION OF TRANSUDATES, EXUDATES AND SEMEN

**Principles.**—1. The excessive accumulation of fluids in the tissue spaces is known as edema while excessive accumulation of non-inflammatory fluids in the serous cavities are designated as *transudates*. The mechanism of their production is similar and essentially due to the fact that instead of there being a perfect balance between the inward and outward flow of fluid through capillary membranes, absorption is exceeded by transudation. Transudates are termed, according to their location, as *hydrothorax* (pleural), *ascites* (peritoneal), *hydrocele* (scrotal), *hydropericardium* (pericardial), *hydroarthrosis* (synovial), etc.

2. *Exudates* are essentially pus in varying degrees of dilution or concentration and usually differ in many important respects from transudates in physical, chemical and cytological properties. But, in some parts of the body and especially in the nose and colon, they must be distinguished from the excessive secretion of mucus due to allergy or other causes.

Since exudates are inflammatory in origin, they are usually due to acute or chronic infections with pathogenic bacteria or animal parasites. On the other hand, they may be caused by irritation or inflammation by sterile substances. Familiar examples are the sterile abscesses produced by the subcutaneous or intramuscular injection of sterile agents; likewise, the peritoneal exudates produced through irritation by blood, bile, pancreatic juice and intestinal obstruction without infection, being similar in origin to "aseptic meningitis" produced by intrathecal injections of sterile sera or other substances. Furthermore, sterile peritoneal and pleural exudates may be due to trauma or to irritation by extension from a nearby infectious process, the latter being similar to "meningitis sympathica" secondary to mastoiditis or sinusitis.

3. Semen is neither a transudate nor an exudate but is, for convenience, discussed in this place. Its examination for spermatozoa is important in appraising the male partner as a factor in any involuntary sterile marriage, as well as for determining the effectiveness of sterilization by vasectomy; also in certain medicolegal situations where paternity is disclaimed on the basis of male sterility and in the examination of stains involving the charge of rape.

### COLLECTION OF TRANSUDATES AND EXUDATES

Transudates and some types of exudates are readily obtained from the serous cavities for laboratory examinations. If for diagnostic purposes only, from 10 to 30 cc. may be obtained by aspiration with a syringe and needle of 16-18 gage. Otherwise, when removed for drainage purposes, larger amounts up to 100 cc. or more should be sent to the laboratory. Since coagulation interferes with some examinations *it is advisable to add 1 cc. of a sterile 2.5 per cent solution of sodium citrate to each 10 cc. of effusion* submitted. This is particularly necessary when exudates are suspected.

1. *Rigid aseptic precautions are required* not only for the protection of the patient against accidental infection, but for the prevention of contamination of the fluid, since bacteriological examinations are essential and routinely indicated. Consequently the skin should be carefully prepared and all instruments and glassware sterilized. The site

should be carefully selected and the skin infiltrated with 1 per cent novocaine solution.

2. To obtain fluid from the *pleural cavity* (paracentesis thoracis) the patient sits upright or has the shoulders elevated on pillows, leaning forward with the arms raised and the hands placed on opposite shoulders. The area of greatest dullness is determined. The site usually chosen is the fifth or sixth intercostal space in the midaxillary line. The sixth to the eighth interspaces below the angle of the scapula are chosen if the fluid is small in amount. The puncture should be made close to the upper border of the rib to avoid the intercostal blood vessels. Fluid should not be removed too rapidly since sudden pulmonary edema or a rapid fall in blood pressure may develop.

3. In obtaining fluid from the *abdominal cavity* (paracentesis abdominis) the site of puncture is in the midline usually about half-way between the symphysis pubis and the umbilicus. The patient should sit up in bed, most conveniently on the edge, with the back well supported by either an assistant or pillows. The bladder should have been emptied before making the puncture. When considerable fluid is present and large amounts are withdrawn, it is advisable not to allow the fluid to escape too rapidly and afterward to adjust an abdominal binder. *Great care is required in cases of tuberculous peritonitis* since the intestine may be adherent to the abdominal wall; indeed, it is frequently safer to make a small incision under local anesthesia.

4. In obtaining fluid from the *pericardial cavity* (paracentesis pericardii) the patient should be placed in the semirecumbent posture. The usual site is the fifth intercostal space below the left nipple, with the needle directed upward, backward and toward the sternum. Some prefer the fifth or sixth interspace close to the sternal border and others the left costoxiphoid notch close to the ensiform cartilage. In other words, the puncture is neither as simple nor as safe as puncture of the pleural and peritoneal cavities. If the needle or trocar impinges against the heart, the movements of the organ will be imparted to the instrument. It is generally wise to make provisions for pericardotomy if there is a question as to the type of effusion present.

5. *Synovial fluid* can be readily obtained with little pain or discomfort. A short needle should be used and care taken not to traumatize the synovial membrane.

## ROUTINE EXAMINATIONS

This usually embraces the following for differential diagnosis as no one method of examination can be used alone; a combination of procedures is advisable.

1. Appearance.
2. Specific gravity, estimated according to the method employed in urine analysis.
3. Presence or absence of partial or complete coagulation and rapidity of coagulation. Exudates coagulate more rapidly and completely than transudates. The latter may not coagulate at all or show flocculi, whereas some exudates (notably those obtained from the lungs by bronchoscopic drainage) may coagulate solid or show partial coagulation.
4. Chemical determinations, especially for quantity of protein.
5. Examination of cells (cytodiagnosis).
6. Bacteriological examination by smear, culture and animal inoculation (in suspected tuberculosis).
7. Complement fixation tests in some instances for tuberculosis, syphilis and echinococcus disease.

## DIFFERENTIAL PROPERTIES OF TRANSUDATES AND EXUDATES

Tests	Transudates	Exudates
Specific gravity .....	1.006 to 1.015 (average about 1.013). Tumor transudates, 1.018 to 1.025	Over 1.018, with average about 1.022
Coagulation .....	Usually absent or slight	Usually positive
Protein .....	Rivalta test usually negative. May be positive after concentration of fluid by absorption. Under 3 per cent in quantity	Rivalta test usually positive. Over 3 per cent in quantity
Cytology .....	Endothelial cells and erythrocytes. Small lymphocytes sometimes predominate. Tumor cells may be found. Eosinophils may be increased after repeated tap-pings	Polymorphonuclears in acute infections. Small lymphocytes in chronic infections. Eosinophils in pneumococcus infections, after repeated tapplings and following artificial pneumothorax. Erythrocytes usually present
Bacteriology .....	Usually sterile. Staphylococcus albus from the skin may occur in cultures	Smears and cultures usually positive for pneumococci, streptococci, etc. Tubercle bacilli in smears and by guinea-pig inoculation
Complement-fixation ....	Positive reactions in syphilis	Positive reactions in tuberculosis and echinococcus disease

## PHYSICAL EXAMINATIONS

**Transudates.**—In *appearance* transudates are usually clear or opalescent and of a light straw or yellowish-green color unless blood is present. A deeper color may be observed in the presence of jaundice. They do not coagulate unless considerable blood is present from accidental puncture of a vein during collection, or from malignant tumors. True chylous transudates (pleural or peritoneal) are milky in appearance due to the escape of chyle from a ruptured or obstructed thoracic duct (filiaris most commonly) and spontaneous coagulation may occur. Pseudochylous or chyloform transudates have the same appearance. They may be relatively clear when first removed, the turbidity and milky appearance increasing upon cooling; some spontaneous coagulation may occur.

2. The *specific gravity* of transudates is usually less than that of exudates. When the former are free of blood, the specific gravity is usually below 1.018. Specific gravity, however, is subject to considerable variation according to the amount of protein present, which is dependent upon variations in the permeability of the capillaries in different parts of the body. Thus, pleural and peritoneal transudates contain more protein and consequently show higher specific gravities than spinal fluid with values as low as 1.005 in the case of subcutaneous transudates or edema fluids.

**Exudates.**—1. Exudates vary greatly in *appearance* according to their origin and consistency. When low in cells they are serous, serofibrinous or hemorrhagic in character and usually indicative of acute virulent infection. Partial coagulation may occur due to the presence of fibrinogen from intense inflammation. When due to infection

with the saccharolytic anaerobic bacilli of gas gangrene, they are apt to be frothy in character. When more highly cellular they may be seropurulent or frankly purulent and creamy, the latter constituting pure pus. When free of blood they vary in color from light yellow to straw but may be greenish or greenish-yellow due to the presence of *B. pyocyaneus*.

2. Not infrequently exudates occurring in the serous cavities contain a mucin-like substance (seromucin) and those occurring in the stools of amebic dysentery are almost entirely composed of a clear, glairy mucus resembling the white of an egg streaked with blood. Exudates occurring on free surfaces, however, may be membranous or pseudomembranous in character.

3. Exudates are usually odorless or of a sweetish odor unless long retained with putrefactive changes. Those due to primary or secondary infection with *B. coli* frequently possess a characteristic fecal odor.

4. Since exudates usually contain much more protein and cells than transudates, the *specific gravity* is generally above 1.018 and may be as high as 1.035.

## CHEMICAL EXAMINATIONS

**Transudates.**— The total *protein* of transudates is usually lower than that of exudates and usually less than 2.5 grams per 100 cc. Pleural and peritoneal transudates due to congestive heart failure, nephrosis, uncomplicated cirrhosis of the liver, etc., may show no more than 0.1 to 1.0 gm. per 100 cc. However, if transudates are present for some time, water may be reabsorbed more rapidly than solids with the result that the protein content is increased and eventually approaches that of an inflammatory exudate. Nevertheless, a determination of total protein is usually of value for differentiating transudates from inflammatory exudates.

2. Albumin usually constitutes the largest part of the protein present in transudates because of the smallness and low viscosity of the molecule, permitting its passage through capillary walls. Small amounts of the globulins may be likewise present, but only rarely appreciable amounts of fibrinogen. Joint fluids, however, usually contain larger amounts of protein and, likewise, a substance closely resembling mucin.

3. The *chloride* content of transudates is somewhat higher than that of the blood plasma, ranging from 720 to 750 mg. per 100 cc. in terms of sodium chloride. The difference is due to the existence of a Donnan equilibrium dependent upon the higher concentration of protein in the plasma as compared with transudates.

4. Transudates contain *glucose* in practically the same concentration as that of the blood. The same is true of *creatinine*, *uric acid* and particularly *urea*. The *calcium* usually ranges from 4.5 to 5.5 mg. per 100 cc. and apparently represents the normal diffusible calcium of the serum; it increases with the protein content due to non-diffusible calcium in combination with protein. Transudates also contain approximately the same amounts of *inorganic phosphorus* as the blood serum, but somewhat smaller amounts of *sodium*, *magnesium* and *potassium*. *Bilirubin* may be present in the pleural and peritoneal transudates of patients with congestive heart failure or cirrhosis of the liver without an increase in the blood; of course it is somewhat increased in the presence of bilirubinemia. As a general rule, determinations of these chemical constituents possess little or no clinical value.



5. *Lipoids* (neutral fat and fatty acids) are not usually present in transudates. A small amount of lecithin, however, varying from 20 to 100 mg. per 100 cc., is usually present. Since capillaries appear to be permeable to cholesterol to about the same extent as to protein, only very small amounts are present in transudates. This is true even in nephrosis, in which the concentration of cholesterol in the blood plasma may be enormously increased.

Of course the milky appearance of chylous transudates is due to the presence of chyle which is rich in finely divided fats; these may amount to as much as 0.05 to 3.85 grams per 100 cc. They cannot be removed by centrifugation but are easily removed by extraction with ether.

The milky appearance of pseudochylous transudates is not due to the presence of chyle but chiefly to lecithin and cholesterol with small amounts of highly emulsified fats derived from fatty degeneration of the cells in the transudate and those lining the pleural or peritoneal cavities. It has also been observed that albumin in a highly dispersed state may impart a milky appearance to such fluids and indeed, relatively large amounts of protein may be present, varying from 0.1 to 4.2 grams per 100 cc. with some spontaneous coagulation. These may occur in lipoid nephrosis and in chronic glomerulonephritis with nephrosis; also in carcinoma of the peritoneum and in tuberculous pleurisy and peritonitis.

6. Methods for quantitative estimations of these substances are the same as employed in blood chemistry examinations.

**Exudates.**—1. In purulent exudates resulting from severe inflammation, as illustrated by empyema, the *total protein* of the serous portion of the fluid, obtained by centrifugation, is generally over 3.0 grams per 100 cc. and may be approximately the same as that of the blood plasma (6.4 to 8.0 gms. per 100 cc.). In the case of exudates resulting from inflammatory processes of lesser intensity, such as tuberculous pleurisy and tuberculous peritonitis, the total protein usually ranges from 0.1 to 0.5 gm. per 100 cc. This protein is due not only to albumin, but likewise to globulins and even fibrinogen, because greatly increased capillary permeability permits the passage of these larger molecules. Pneumococcal exudates appear to be particularly rich in fibrinogen, which accounts for the frequency with which they show spontaneous coagulation.

2. The *glucose* content, however, is usually much lower than that of transudates, due to the destruction or glycolysis of this sugar by the action of bacteria and cells, the degree of reduction being dependent somewhat upon the intensity of the inflammatory process.

3. The *chloride* content is likewise usually lower than that of transudates and approaches that of blood plasma, with the degree of reduction varying roughly with the increase of protein in accordance with the laws governing the concentration of readily diffusible substances on two sides of a semi-permeable membrane under such circumstances. The chloride content of pleural exudates in pneumococcal pneumonia is particularly low because of the low chloride concentration of the blood plasma in this disease.

4. *Creatinine*, *uric acid* and particularly *urea* are present in practically the same concentrations as in the blood. *Cholesterol* is likewise practically always present, particularly in exudates of long standing, being probably derived from degenerative changes either in the cells present in the exudates or of those lining serous and abscess

cavities. This is apparently due to the fact that capillaries have about the same permeability for cholesterol as for protein, so that the lipid content of exudates is roughly parallel to their protein content. The cholesterol content, however, may decrease markedly following repeated tapping, values ranging from 1 to 4.5 gm. per 100 cc. falling to 20 to 50 mg. per 100 cc. In some cases, showing large amounts of cholesterol, *fat* is also present and particularly in tuberculous pleural and peritoneal exudates.

5. The *calcium* content is generally higher than that of transudates due to a non-diffusible fraction, which is probably in combination with protein; the same is likewise true of *magnesium* because of increased protein concentration.

6. Methods for quantitative estimations of these substances are the same as employed in blood chemistry examinations.

**Rivalta Qualitative Test for Protein.**—1. Place 150 cc. of distilled water in a conical flask.

2. Add 0.1 cc. of glacial acetic acid.

3. Mix thoroughly.

4. Allow 1 or 2 drops of the puncture fluid to fall into this weak acid solution.

5. A distinct cloud will be observed in the wake of the falling drop if the fluid is an exudate. As a rule, if the fluid is a transudate no turbidity will be noticed. The reaction is probably due to the large amount of a mucin-like substance called sero-somucin, especially likely to occur in exudates. Positive reactions may occur with transudates concentrated by absorption, or those developing after tapping, and the production of artificial pneumothorax.

**Quantitative Test for Protein.**—1. The protein content may be determined by the methods employed for the quantitative estimation of protein in urine.

2. Owing to relatively large amounts of protein likely to be present, it is advisable to test the fluid diluted 1:2, 1:5 and 1:10 with saline solution. The measure of precipitate is multiplied by the dilution factor.

3. The results show grams per liter. Divide by 10 to obtain the per cent.

## CYTOLOGICAL EXAMINATIONS

**Principles.**—1. *Transudates* are characterized by the fact that they contain but few cells in contrast with inflammatory exudates in which they are very numerous. Those commonly formed are of mesothelial types comprising large cells, with abundant cytoplasm, and containing one, sometimes two, round or oval, palely staining nuclei. An occasional lymphocyte may be found, but only a few neutrophils or monocytes. A few erythrocytes are also commonly found, due to puncture, but cytological examinations are hardly worth while when macroscopic amounts of blood are present, except when malignancy is suspected. In carcinoma, mesothelial cells predominate, but are accompanied by numerous lymphocytes and erythrocytes. Cancer cells cannot be recognized in stained smears of sediment secured by centrifuging a portion of the transudate, but the presence of numerous mitotic figures is suggestive. Under these circumstances it is advisable to centrifuge large amounts of transudate and to prepare sections of the sediment according to the method of Mandelbaum, described many years ago. Examination of these by expert histopathologists has proven valuable in the diagnosis of malignant tumors involving the pleural and peritoneal cavities. Malignant cells or fragments of tissues are found in a little over 50 per cent of cases.

Carcinoma is more readily detected than sarcoma. Of course negative findings do not exclude malignant disease.

2. The total cells of *exudates* are always higher than that of transudates. They may be counted in noncoagulated fluids by the same technic as employed in counting the leukocytes of the blood. In serous exudates the counts may be as low as 200 to 500 per c.mm. or as high as 4000 to 40,000 or more per c.mm. in the case of sero-purulent and purulent exudates.

3. A differential cell count or *cytodiagnosis*, however, is of far more clinical value and should be included in all routine examinations. These may be made with simple means of exudates or of sediment stained by the method of Gram, or in the same manner as blood smears. Supravital methods usually reveal more details of value in differentiating the cells present.

4. Early or moderately advanced exudates due to infection with the pyogenic microorganisms, invariably show a great preponderance of neutrophils. They are mostly well preserved and many are phagocytic with usually less than 10 per cent mesothelial cells. In late exudates the neutrophils likewise predominate, with increased phagocytosis, but with varying degrees of degeneration of the cytoplasm and nuclei; about 20 per cent of mesothelial cells are present in varying stages of degeneration and sometimes difficult to recognize.

5. In tuberculous exudates (pleural, peritoneal, etc.) small lymphocytes greatly predominate along with monocytes and a few neutrophils. The latter may be quite numerous in the early stages.

**Cytodiagnosis.**—1. Centrifuge *fresh* (important) specimen of the fluid. To prevent coagulation, the fluid may be collected in a little sodium citrate solution although cytodagnosis is better made without the use of anticoagulants.

2. Pour off supernatant fluid and make thin smears of sediment on slides. It is essential to use packed sediment in order to secure sufficient cells in smears.

3. Dry in the air.

4. Stain with Wright's or Giemsa's stain according to the method of staining blood smears.

5. Count and tabulate at least 100 of the cells. Four types may be present: lymphocytes, neutrophils, eosinophils and endothelial cells. Erythrocytes in varying numbers are usually present in all fluids.

(a) *Neutrophils* predominate in acute infectious processes, especially those due to the pyogenic organisms (Fig. 124), and may be found in early acute cases of serous tuberculous exudates.

(b) *Lymphocytes* predominate in chronic processes, especially those due to tuberculosis and syphilis (Fig. 124). They may also predominate in some chronic non-tuberculous pleurisy, chronic transudates, or even tumor transudates.

(c) *Eosinophils* may be present, but have no clinical significance except to suggest an allergic origin of the fluid or disease due to an animal parasite. Serous effusions caused by pneumococci may contain as high as 10 per cent eosinophils, and, according to Foord, a frequent reason for their presence is repeated aspirations when they may be as high as 75 per cent. Differential cell counts with smears of nasal secretions for eosinophils are helpful in the diagnosis of allergic coryza.

(d) *Endothelial cells* (Fig. 124) in large numbers along with lymphocytes and erythrocytes are usually present in transudates and are largely derived from the endo-

thelium lining the large serous cavities (pleural, peritoneal, pericardial). They commonly occur in sheets as well as singly.

(e) The presence of masses of large cells, irregular in size and shape, often vacuolated, showing prominent nucleoli and sometimes mitotic figures, is highly suggestive of malignancy, but definite diagnosis is better made by sections of the imbedded sediment, in which fragments of tumor tissue, especially gland acini in adenocarcinomata, can be sometimes found. Confusion may result in smear examinations when degenerated forms of large mononucleated cells, either serosal desquamations or cells of other types ordinarily designated as macrophages, are seen.

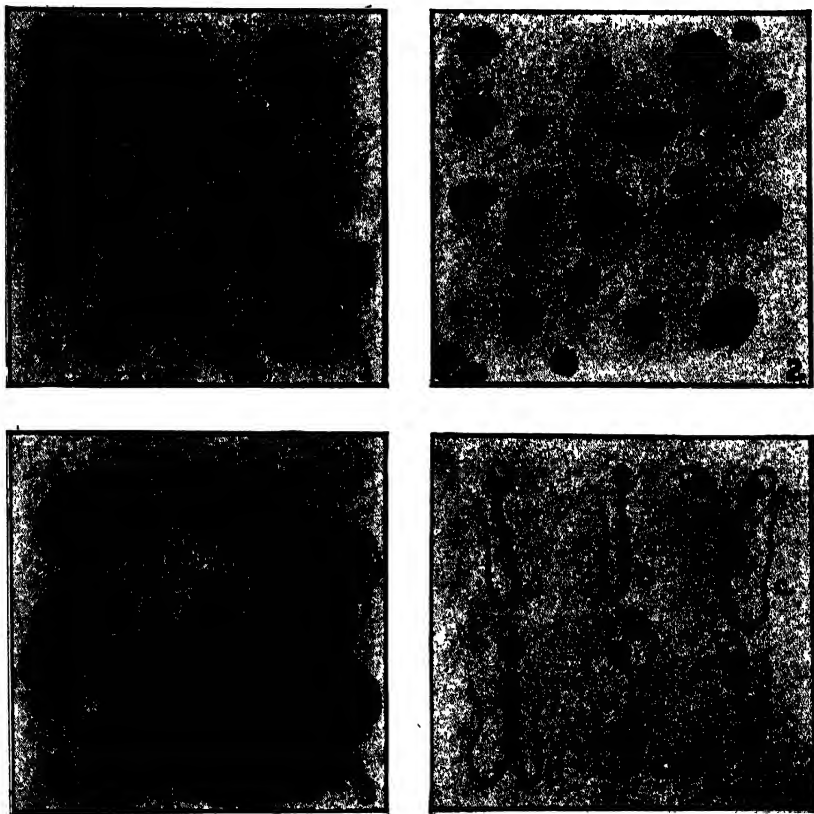


FIG. 124.—CYTOLOGY OF TRANSUDATES, EXUDATES AND SEMEN

1, Excess of neutrophils and macrophages; 2, excess of lymphocytes; 3, excess of endothelial cells; 4, spermatozoa (*a*, normal; *b*, pin head; *c*, double head, double body; *d*, double tail; *e*, double nucleus; *f*, irregular body).

**Mandelbaum's Cytodiagnostic Method for Effusions.**—1. Place the fluid in a large Erlenmeyer flask in the refrigerator for 12 to 18 hours.

2. Decant the supernatant fluid and transfer the sediment to a 50 cc. centrifuge tube which has a tapered bottom.

3. Centrifuge at moderate speed for 20 minutes.

4. Decant the supernatant fluid and discard.

5. Fix the sediment with 10 per cent formalin for 24 hours.

6. Treat the fixed sediment as a tissue running it through the alcohols and imbedding in paraffin.

7. Cut thin sections from above downward to include all of the cellular elements, which may not lie either at the top or the bottom.

8. Stain the sections with eosin and hematoxylin or iron hematoxylin. Bits of malignant tissue may be definitely demonstrated by this method in a large percentage of cases.

**Method for the Histological Examination of Transudates (Modified after Stanley).**—1. The fluid should be prepared as soon as possible after removal. In case of delay, add an equal volume of 10 per cent formalin to fix the cells.

2. Thoroughly centrifuge a large amount (250 to 500 cc.) in 50 cc. centrifuge tubes.

3. Wash the sediment from each tube with 10 per cent formalin into a small flat bottomed tube (10 cc. homeopathic vial may be used) and centrifuge thoroughly to pack the sediment. Allow to stand for 1 or 2 days and decant the formalin.

4. Add 70 per cent alcohol for 24 hours and centrifuge. Repeat in same manner with 95 per cent alcohol.

5. Add absolute alcohol and place in the paraffin oven at 55° C. for about 3 hours, changing the alcohol once or twice.

6. Remove the alcohol and add chloroform. Gently loosen the sediment so that it floats. Place in oven for 30 minutes.

7. Remove chloroform and add paraffin at 55° C. changing every half hour for 2½ hours.

8. Allow the last paraffin to harden in the tube in a refrigerator. Then heat the tube gently to loosen the paraffin slightly and remove the block (the tube may be gently broken if necessary).

9. Trim, mount on block, section and stain.

### BAACTERIOLOGICAL EXAMINATION

1. These methods are described in more detail on pages 415 to 416.

2. Cultures should be made on blood agar or in hormone broth. Relatively large amounts of fluid, like 1 cc., should be employed.

3. Smears of sediment should be stained by the Gram method and for tubercle bacilli.

4. Prolonged microscopic examination is usually required for the detection of tubercle bacilli.

5. In conducting the inoculation test for tubercle bacilli, at least several ounces of fluid should be centrifuged and the sediment injected into guinea-pigs. The injection of 5 cc. amounts of fluid may yield falsely negative results.

### METHODS FOR THE EXAMINATION OF SEMEN

**Principle.**—The purpose of the examination of semen is to determine whether, in cases of sterility, the cause is due to quantitative or qualitative changes in the spermatozoa.

**Collection of Semen.**—1. A rubber condom is thoroughly washed under running water and dried before use.

2. After ejaculation the semen is transferred immediately from the condom to a clean glass container such as sputum bottle or test tube. This facilitates transportation and prevents any deleterious effect of the rubber on spermatozoa, which is particularly the case when the condom contains spermicidal substances. The container should be tightly closed with a cork stopper and labeled with name, date and hour of emission.

3. The specimen should be delivered to the examiner as quickly as possible. Precautions to keep the sample at 37° C. are unnecessary and may even prove harmful if patients, told to keep the specimen warm in a thermos bottle, do not measure the exact temperature. Furthermore, motility lasts longer at lower temperature, allowing more time for transportation.

**Macroscopic Examination.**—1. Measure the volume of semen in a small graduated cylinder. The amount varies between a few drops up to 10 cc. There is quite a variation in amount in different samples from one donor, depending on the period of continence preceding the examination. The average lies between 3 and 4 cc. Samples amounting to less than 1.5 cc. are considered below normal, though sterility could not be ascribed to this fact alone unless other deficiencies are recognized in the same specimen.

2. Note the viscosity of the sample. Freshly ejaculated semen acquires a high degree of viscosity but self-liquifaction takes place and should be completed after 30 minutes. The absence of liquifaction may inhibit the movement of spermatozoa thereby interfering with fertilization.

3. The determination of the pH is of little value. It is always found to be on the alkaline side with a range from 7.2 to 8.9, the average being 7.8. Abnormalities never are accompanied by a fall below 7.2.

**Motility of Spermatozoa.**—1. Place a drop of semen on a slide and cover with a coverglass or prepare a hanging drop. Examine with low and high dry objectives. The following should be noted:

*Azoospermia*: absence of spermatozoa (this should be confirmed after centrifugation of specimen).

*Oligozoospermia*: only a few motile spermatozoa found.

*Necrozoospermia*: spermatozoa are present but immobile.

Observe also the presence of cells other than spermatozoa, *i.e.*, testicular cells, epithelial cells, leukocytes or erythrocytes. The presence of crystals should also be noted.

2. If motile spermatozoa are found, note roughly the proportion of motile to immobile cells. For this determination the following technic is recommended: A disc of black paper is fitted in the eyepiece of the microscope after cutting a small slit or square in the middle. This limits the field of vision and thus simplifies the counting of the spermatozoa and decreases errors introduced by the constant change of the sperm population in the field. Immobile spermatozoa are counted after the motile ones.

3. Ten to 15 per cent of immobile spermatozoa are encountered in fertile samples. If the motility is very low, repeat the examination after heating the sample to 37° C. This frequently restores motility.

4. A differentiation between "locomotoric" and "stationary" or other grades of motility is without great value since spermatozoa are known to stick to interfaces

such as the surface of the drop or the coverglass, or they may attach themselves to formed elements such as cells. Furthermore, the viscosity of the sample may influence the motility. There is considerable individual variation in the ability of spermatozoa from different specimens to remain motile, which partly depends on the temperature at which the sample is kept. At 37° C. all spermatozoa usually are immobilized after 8 hours while at 4° C. a number of motile cells may be encountered after as many as 4 days or more. To determine the viability of a specimen it is, therefore, advantageous to keep it at 4° C. and to determine the motility in samples taken after 6, 12 and 24 hours.

**Counting of Spermatozoa.**—The number of spermatozoa in a sample of semen are counted in the same manner as described for leukocytes except for the diluent.

1. Mix the specimen thoroughly by very gently shaking or stirring with a glass rod.

2. Using a leukocyte counting pipet, draw the semen up to the 0.5 mark and the diluent to the mark 11:

#### DILUTING FLUID

Sodium bicarbonate .....	5 gm.
Formalin .....	1 cc.
Distilled water .....	100 cc.

*Note:* The bicarbonate in the diluent counteracts mucus and the formalin immobilizes the spermatozoa. When the specimen is very viscous due to an excess of mucus, 1 cc. of semen in 19 cc. of diluting fluid thus omitting the use of the leukocyte pipet. In case the number of spermatozoa in a sample proves to be very small, it will be necessary to draw the seminal fluid to the mark 1.0 in the leukocyte pipet, or 1 cc. is added to 9 cc. of the diluent.

3. Fill the chamber of the hemocytometer with the diluted specimen, count and calculate as described on page 67 for counting leukocytes. The result is expressed in the number of spermatozoa per cubic centimeter. It is therefore necessary to multiply the result by 1000, since leukocytes are calculated in cells per cubic millimeter.

4. The normal semen contains an average of 100 to 150 million spermatozoa per cubic centimeter. The lower the count below 60 million the less likelihood of fertility. However, no diagnosis of sterility is justified unless other abnormalities are found in the sample.

**Method for Staining Seminal Smears.**—1. Take a clean slide and prepare a thin smear of semen.

2. Dry in air and fix by heat.

3. Add 1 per cent chloramine for several minutes to remove excess mucus.

4. Wash with water followed by 95 per cent alcohol. Dry by blotting on filter paper.

5. Stain for 2 to 5 minutes with the following:

Ziehl Neelsen's carbol fuchsin .....	2 parts
Conc. alcoholic sol. of eosin .....	1 part
Alcohol 95 per cent .....	1 part

6. Wash with water and counterstain with Loeffler's methylene blue for a few seconds.

7. Wash, dry and examine under oil immersion.

8. The heads of the spermatozoa show a purplish color while the tail and middle piece are red.

**Morphological Examination for Immature and Abnormal Spermatozoa.**—

The purpose of this examination is to discover abnormal forms of spermatozoa and to estimate their proportion to normal cells. Cellular constituents other than spermatozoa should be noted. Stained smears are employed.

1. Count the number of spermatozoa in a microscopic field without regard to their morphology.

2. The same field is searched for immature forms of spermatozoa. The different developmental stages may be encountered in pathological semen but are rare in fertile specimens.

3. Examine 100 to 500 spermatozoa for abnormalities (Fig. 124) referable to the *heads* (which may be too small or too large, pointed or with ragged edges, show an atypical distribution of chromatin, the presence of acidophil vacuoles or double heads); the *middle* pieces (which may be absent, bifurcated, swollen, etc.) and the *tails* (which may be double, curled, rudimentary or absent).

4. Semen containing up to 20 per cent abnormal spermatozoa is still considered fertile. The higher the percentage of abnormal cells above this arbitrary value, the more doubtful the fertility.

5. Finally, examine for cells other than spermatozoa, *i.e.*, epithelial cells, leukocytes and erythrocytes. Also crystals may be found in abundance. However, the age of the sample has to be considered since numerous crystals may be formed in normal semen upon standing.

**Biometrical Evaluation of Spermatozoa.**—This study has the objective of measuring the length of the head of a large number of spermatozoa for the establishment of a distribution curve. While in fertile cases this curve shows the high peak and small base of a "normal" distribution curve it takes on an irregular and flatter shape with broader base when infertile cases are examined. It is this irregularity or flatness of the distribution curve which is significant, and not the average head length in the individual samples. This may vary in fertile samples between 3.8 and 5.4 microns. The method is as follows:

1. A stained seminal smear is prepared according to the method described.

2. The slide is focused under oil immersion. The magnification should reach at least 1500 $\times$ . The microscope should be fitted with a mechanical stage.

3. An inclined mirror is fixed to the eyepiece of the microscope to project the image onto a screen consisting of a revolving plate of 10-inch diameter. The screen is covered with white graph paper in millimeter scale (added enlargement 3000 $\times$  total).

4. The individual spermatozoön is measured by turning the screen in such a way that one of the lines of the graph paper parallels the axis of the cell. The length of the head can be read off directly from the paper.

5. Three hundred cells are measured in such a way and after grouping of the heads showing approximately equal length, the distribution curve is plotted, or the coefficient of variation is calculated. The coefficient of variation will be greater in infertile samples than in fertile samples.

**Detection of Semen on Materials for Medicolegal Purposes.**—In medicolegal cases seminal stains on materials such as clothing, linen, etc., may be identified by



demonstrating the presence of spermatozoa, or by microchemical reactions such as the Florence test. Hektoen and Rukstinat have reported upon the use of a precipitation test using material from such stains as antigen. They showed also that the blood group of the donor might be determined.

*Demonstration of Spermatozoa.*—1. A small piece of the soiled material, not more than  $\frac{1}{2}$  inch in diameter, is placed on a clean slide. It is wetted with a few drops of saline solution and the surface scraped off with a scalpel. The few drops of fluid are then spread over the slide and after drying the film is stained according to the method described.

2. Examine the slide for the presence of spermatozoa and note any abnormal forms; these may be helpful in identifying a sample since the semen from a given individual may present characteristic abnormalities.

*Microchemical Method (Florence Test).*—In cases where an azoospermia is present, no spermatozoa can be obtained from suspected stains, but a positive microchemical test will be a strong suggestion that the stain in question is of seminal origin.

1. Soften the material with water and place upon a slide.
2. Add a few drops of reagent:

#### REAGENT

Iodine .....	2.54 gm.
Potassium iodide .....	1.65 gm.
Distilled water .....	30.0 cc.

3. Examine at once with the medium power of the microscope.
4. If the stain is produced by semen there will be found dark brown crystals in the form of rhombic platelets which resemble hemin crystals, or of needles, often in clusters.
5. These findings are not absolute proof of the presence of semen as some other substances give the same reaction among which are crushed insects and extracts of various organs. The reaction will occur even though the semen is several years old.

## METHODS FOR THE COLLECTION AND EXAMINATION OF CEREBROSPINAL FLUID

**Principles.**—1. The cerebrospinal fluid is normally produced by the highly vascular choroid plexus in the ventricles of the brain by a process of filtration from the blood plasma through a selectively permeable membrane. But since, under normal conditions, the hydrostatic pressure of the capillary blood is believed to be greater than that of cerebrospinal fluid, it is evident that ultrafiltration alone does not account for its formation and that it may be, at least in part, a secretion of the cells of the choroid plexes. The perivascular spaces and the ependymal cells of the ventricles and the spinal canal may also participate to some degree in its production but there is no evidence of any secretory activity of the arachnoid villi.

2. In acute and chronic congestion of the meninges the volume of cerebrospinal fluid is increased, not only by increased permeability of the choroid plexus, but from increased transudation of plasma through the capillaries. Likewise, in acute and chronic meningitis it is increased by the production of inflammatory exudates.

3. Filtration by the choroid plexus and the meninges is highly selective. The chemical constitution of normal cerebrospinal fluid is essentially that of Locke's solution plus small amounts of glucose and plasma proteins. However, chlorides, magnesium, sodium and carbon dioxide of the plasma pass very freely.

Albumin, globulins, urea, creatinine, amino acids, uric acid, lactic acid, glucose, acetone, lipase, amylase, and such inorganic substances as calcium, potassium, phosphates, sulfates and iron pass to some extent due to partial permeability. However, when some of these are greatly increased in the plasma, increased amounts are commonly found in the cerebrospinal fluid, especially glucose, urea and chlorides.

On the other hand, bilirubin, fibrinogen, complement, natural antibodies (antitoxins, agglutinins, opsonins, etc.) and cholesterol do not pass at all or but in minute amounts. Even when these are greatly increased in the plasma by traces, or none at all, occur in the cerebrospinal fluid providing the choroid plexus and meninges are not involved.

Insofar as foreign substances are concerned, only alcohol passes freely although chloroform, urethane and hexamethylenamine pass to a considerable extent. Arsenic, lead, mercury, bromides, iodides, salicylates, strychnine, the sulfonamides and toxins do not pass at all or but in very minute amounts. But permeability is commonly increased to some extent in acute and chronic meningitis and especially through a breakdown in the barrier of the choroid plexus. Thus, in neurosyphilis, the pentavalent arsenical compounds and bismuth as well may pass to a considerable extent as well as some of the sulfonamides in acute meningitis with special reference to sulfanilamide. Naturally this has an important bearing upon the treatment of acute and chronic infections of the central nervous system.

### COLLECTION

Cerebrospinal fluid is usually collected by spinal puncture. Cisternal puncture is advocated by some because of its technical ease and greater freedom from pain and puncture headache, but is not recommended except in cases of subarachnoid block due to purulent exudates or other causes.

**Indications and Contraindications.**—Aside from the use of spinal puncture and drainage for therapeutic purposes, examinations of the cerebrospinal fluid are indicated as indispensable aids in establishing etiological diagnosis not only in all cases of known or suspected acute and chronic meningitis due to infection, but in the diagnosis of so-called "serous meningitis" or "meningismus" and aseptic meningitis as well. Also in all cases of chronic syphilis for the detection of asymptomatic or symptomatic infection of the central nervous system as well as in relation to the treatment of the disease. Indeed no case of syphilis can be regarded as cured unless at least one examination of the cerebrospinal fluid has shown a normal pressure, normal total cells and negative protein, Wassermann and colloidal gold reactions.

Spinal fluid examinations are also indicated as aids in the diagnosis of cerebral edema, multiple sclerosis, acute anterior poliomyelitis, lethargic and other types of encephalitis, tumors and abscesses of the brain, tumors of the spine or spinal cord, cerebral hemorrhage, hemorrhage due to trauma and in cases of coma of unknown etiology.

Many authors list a considerable number of contraindications to spinal puncture with special reference to cerebral hemorrhage and tumors in the posterior fossa. But the danger of increasing hemorrhage by reducing spinal fluid pressure is so slight, when the minimal amount of fluid is *slowly* withdrawn, that the operation is seldom contraindicated. The same is true in all cases of increased intracranial pressure with papilledema, providing the fluid is removed very slowly, with a careful watch for respiratory distress which may be due to herniation of the medulla or cerebellum in the foramen magnum. For this reason a spinal or cisternal puncture should not be done at all or only with extraordinary precautions in suspected tumors of the posterior fossa of the skull.

It is sometimes stated that spinal puncture should not be done in the presence of septicemia because of the danger of favoring the localization of microorganisms from the blood in the meninges. Experimental and clinical investigations, however, have not shown this to occur unless, possibly, when some substance is injected at the same time capable of producing an aseptic meningitis with a breakdown of the barrier of the choroid plexus. The same is true in suspected localized meningitis providing only small amounts of fluid are slowly removed.

Accidents have happened in connection with spinal puncture due to injury of the cord, infection, or other causes, but clinical experience has amply proven that the operation is so safe, when properly conducted, that it should never be omitted when examinations of cerebrospinal fluid are required for diagnostic or, its drainage, for therapeutic purposes. It is true that spinal puncture headache may follow in a small percentage of cases, even under the best of conditions and especially in ambulatory cases, but the information to be gained usually far outweighs the temporary incapacity and discomfort with no after effects at all.

**Technic.**—1. Spinal puncture for the collection of cerebrospinal fluid may be conducted in an office or laboratory, but is better done in a hospital or the home of the patient, since it is advisable for the patient to rest in bed for at least 18 hours immediately after the puncture as a safeguard against spinal puncture headache.

2. The *needle* should not be too large, in order to reduce pain to a minimum and to inflict the minimum of damage to the meninges. Gage No. 19 is about right, unless acute suppurative meningitis is suspected, in which case No. 15 may be used if a

purulent and thick fluid is present. The needle should be sterilized just before use and should be perfectly straight and sharp with a short bevel. Crooked, rusty, dull and unnecessarily large needles are the usual causes of failure and the infliction of unnecessary pain.

3. The *sitting posture* may be used in the puncture of ambulatory adults, as shown in Figure 125, but the reclining posture with the patient lying on his right side (Fig. 126) is recommended, especially if the spinal fluid pressure is to be taken. The latter is required in the case of children and sick adults.

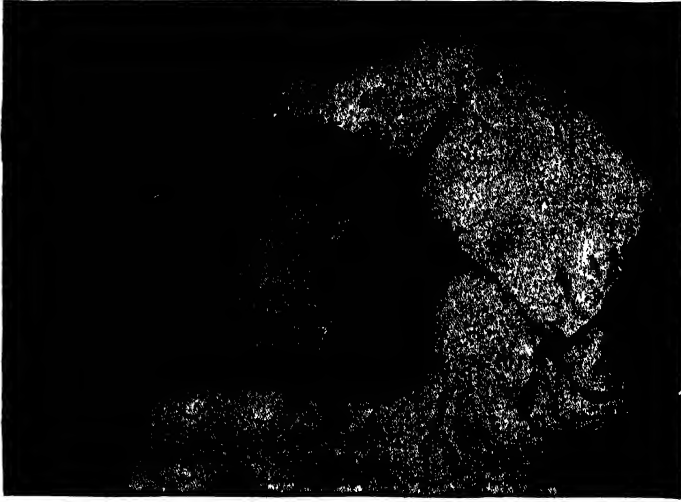


FIG. 125.—SPINAL PUNCTURE IN THE SITTING POSTURE  
(From Kolmer in *Keen's Surgery*, W. B. Saunders Co., Philadelphia.)



FIG. 126.—SPINAL PUNCTURE IN THE PRONE POSITION WITH THE BACK WELL ARCHED AND PERPENDICULAR TO THE TABLE

(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

4. The skin should be carefully disinfected with tincture of iodine followed by alcohol. The hands of the operator should be likewise carefully cleansed and the use of sterile rubber gloves is recommended. The operative field should be protected with sterile sheets and towels.

5. With adults the puncture can usually be made without an anesthetic. The skin may be infiltrated with sterile 1 per cent novocaine or butyn solution (Fig. 127). Struggling children and adults may require a few drops of chloroform as it is dangerous to conduct the puncture under such conditions since the needle may be broken.



FIG. 127.—PRODUCING LOCAL ANESTHESIA

(From Kolmer in *Keen's Surgery*, W. B. Saunders Co., Philadelphia.)

6. Puncture is best conducted between the fourth and fifth or between the third and fourth lumbar vertebrae.

7. The "soft spot" between the spinous processes is located and the needle *gently and slowly* passed in the middle line. The distance varies according to the age and weight. A peculiar "give-way" sensation to the needle denotes entrance into the subarachnoid space, or during its passage the stylet may be removed from time to time to determine whether or not it has entered sufficiently as shown by flow of fluid.

8. If pure blood is obtained, the needle should be withdrawn and the needle cleansed or the puncture repeated with a fresh needle.

9. If there is no flow of fluid the needle may be gently turned or slightly withdrawn or entered a little further. "Dry taps" are usually due to the fact that the needle has not entered the subarachnoid space.

10. The pressure (if to be taken) should be taken before the escape of fluid (Fig. 128).

11. *Fluid should be collected in two sterile tubes*, one of which (No. 2) may contain a trace of powdered potassium oxalate to prevent coagulation. From 3 to 5 cc. may be collected in No. 1 to be used for culture and the Wassermann test even if it is slightly blood-tinged. A similar amount may be collected in No. 2 to be used for the total and differential cell counts, protein and sugar determinations and the colloidal tests (gold, mastic or benzoin). This fluid should be free of blood.

12. The needle is now quickly withdrawn, the iodine removed from the skin

and the puncture sealed with flexible collodion or with an aseptic dressing. The patient should rest on the back for at least half an hour and preferably stay in bed for at least 18 hours to reduce the chances of developing spinal puncture headache, which is believed to be due to the continued leakage of spinal fluid into the epidural space (hence the advisability of using as small a needle as possible and of reaching the fluid at the first puncture).



FIG. 128.—MEASURING SPINAL FLUID PRESSURE WITH A MERCURY MANOMETER  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

### ORDER OF ROUTINE EXAMINATIONS

**When Meningitis is Known or Suspected.**—1. Record physical characteristics (color, transparency, coagula and sediments).

2. Then make a culture before any chances of contamination occur. Inoculate media (blood agar; glucose hormone broth, etc.) with at least 0.5 and preferably 1 cc.

3. Shake well and do total cell count.

4. Centrifuge and prepare smears of sediment for cytological examination; stain smears by Gram method for meningococcus, streptococcus, pneumococcus, *B. influenzae* or other organisms. Stain smears for tubercle bacilli if tuberculous meningitis is suspected (also smears of coagula and inoculate guinea-pigs).

5. With supernatant fluid conduct tests for protein, sugar and chlorides (if requested).

6. Report the findings at this point.

7. Follow with a report on the cultural findings. A colloidal gold test may be conducted but is not necessary. The Wassermann reaction is not required.

**When Syphilis Is Known or Suspected.**—1. Record physical appearance.

2. Make an *accurate* total cell count as soon as possible. A differential is not ordinarily required.

3. Conduct a qualitative test for protein (Pandy preferred).

4. Conduct Kolmer-Wassermann or flocculation tests.

5. Conduct colloidal gold (preferred), mastic, or benzoïn tests.

6. Bacteriological examination is not required.

**When Acute Poliomyelitis or Encephalitis Are Known or Suspected.**—

1. Record physical appearance.

2. Make accurate total cell count as soon as possible.

3. Centrifuge thoroughly and make cytological examination of sediment.

4. Test supernatant fluid for protein and sugar.

5. Bacteriological and Wassermann tests are not ordinarily required.

### AMOUNT AND PRESSURE

Considerable information of diagnostic value is to be gained at the bedside by manometric determinations of the pressure of the fluid.

**Amount.**—The total amount of cerebrospinal fluid has been variously estimated but satisfactory data in relation to age and body weight are not available. For the adult, it is thought to vary from 100 to 150 cc. which corresponds roughly to about 1 cc. per pound of weight. Under the circumstances the withdrawal of 5 to 10 cc. for diagnostic purposes is harmless and especially since it is replaced rapidly.

**Pressure.**—1. The pressure of the cerebrospinal fluid under normal conditions has been stated to vary from 60 to 200 mm. of water or from 0 to 8 mm. of mercury when the individual is in the recumbent position and perfectly quiet. The average is commonly placed at 100 to 200 mm. of water. Pressure between 200 to 250 mm. are suspiciously increased and definitely abnormal over 250 mm. It is not possible to define the limits of abnormally low pressure except to state that 90 mm. or less may be so regarded. There are no differences in relation to sex and apparently no great variations in relation to age; it is to be noted, however, that pressure readings in infants and young children are notoriously unreliable because complete physical and emotional relaxation are most difficult to attain. The fluid usually flows from the needle at the rate of about 1 drop per second but calculations made on this basis are notoriously inaccurate. The pressure is almost doubled in the sitting position and averages about 200 mm. of water. Crying, coughing, sneezing, excitement, emotional states and general anesthetics raise the pressure, presumably by increasing the size of the capillary bed in the brain and meninges. Recent investigations have indicated a reciprocal relationship between blood and spinal fluid volumes with an "elastic" factor which takes into consideration a component depending on the ease of vascular adjustments as well as the distensibility and collapsibility of the meningeal sac.

2. As a general rule it is advisable to measure the pressure before the withdrawal of fluid; again after the withdrawal of 5 cc. with a final reading after the withdrawal of 10 cc. Normally the pressure falls 30 to 50 mm. of water. A drop of less than 20 mm. is suggestive of a large reservoir, as in hydrocephalus or "serous meningitis", while an excessive drop is suggestive of a small reservoir, as in loculation of the fluid below a cord tumor.

3. The intravenous injection of large amounts of isotonic solutions causes a temporary rise in pressure; oral administration may have the same effect. The intravenous injection of a hypertonic solution, as a 30 per cent solution of sodium chloride, produces a reduction in pressure persisting for long periods; this has been found to facilitate operations on the brain by causing its shrinkage and thereby preventing extension through trephine openings.

**The Ayala Quotient.**—This is determined by taking the initial pressure, withdrawing 10 cc. of fluid, and taking the final pressure expressed as follows:

$$\frac{10 \text{ cc.} \times \text{final pressure}}{\text{initial pressure}}$$
 Normal values are 5.5 to 6.5. A value of 7 or over indicates a large reservoir as in hydrocephalus or "serous meningitis" while values below 5 indicate a small reservoir.

**Queckenstedt Test.**—It has long been known that constriction of the neck increases spinal fluid pressure providing there is no block of the subarachnoid space. However, Queckenstedt first analyzed the significance of jugular compression in causing increased intracranial pressure which may be detected immediately by an increase of spinal fluid pressure. This test should always be done whenever complete or partial subarachnoid block or lateral sinus thrombosis is suspected. In complete subarachnoid block the normal rise in pressure after compression of the jugular veins fails to occur; in partial block it is delayed and partial. In thrombosis of a lateral sinus compression of the jugular on the affected side causes no rise in pressure, while compression of the unaffected side alone gives the same rise as one would expect from compression of both jugular veins.

## PHYSICAL EXAMINATIONS

**Appearance.**—The normal cerebrospinal fluid is transparent, colorless and of crystalline clarity with a specific gravity of 1.006 to 1.008 and a freezing point of  $-0.551^{\circ}$  to  $-0.558^{\circ}$  C.

As previously stated, it is advisable to collect the fluid in 2 test tubes as the first flow may contain a small amount of blood from the puncture which renders the fluid hazy or cloudy. Otherwise, the normal fluid has the appearance of distilled water. The physician should always inspect it very carefully at the bedside and preferably against a dark background. If the presence of blood may be excluded, any departure from perfect clarity is to be regarded as abnormal. On the other hand, however, *perfectly clear fluids may be pathological*, as is almost invariably true in syphilis and of frequent occurrence in tuberculous meningitis, poliomyelitis, lymphocytic choriomeningitis and encephalitis.

When distinctly hazy or of a ground-glass appearance, the total cell count is increased to at least 300 to 700 leukocytes per c.mm. (pleocytosis) and if meningitis is suspected clinically, the physician is justified in immediately instituting treatment on this basis until further diagnostic data are supplied by the laboratory. Higher total cell counts, along with the presence of bacteria, render fluids faintly turbid, markedly turbid or frankly purulent.

**Coagula and Sediments.**—Normal spinal fluid does not form coagula, pellicles or sediments upon standing. Under abnormal conditions, however, coagula and pellicles may form due to the presence of fibrinogen, although *their absence does not*



*exclude the possibility of fluids being otherwise pathological.* The time required for their formation varies in different diseases. Thus, in suppurative meningitis, a coagulum may form in a very short time while in tuberculous meningitis 12 to 24 hours may be required.

Coagula may occur as numerous, small flocculi without pellicles, as in syphilitic fluids, as "cobweb" or "pine-tree" coagula with pellicles suspended from the surface of the fluid in tuberculous meningitis, or as heavy coagula with sediments sinking to the bottom of the test tube, as seen in acute purulent meningitis. The presence of small amounts of fresh blood intimately mixed with the fluid does not produce coagulation. Under the conditions, if blood is absent, coagulation is always abnormal.

**Color; Syndrome of Froin.**—1. Normal spinal fluid is colorless. In acute purulent meningitis it may be grayish, as in pneumococcal and streptococcal meningitis, or yellowish-green, as in meningococcal meningitis.

2. The most characteristic color change is that designated as *xanthochromia* (yellow) which may be due to the presence of various pigments. For example, a frequent cause is the presence of altered hemoglobin (hemorrhagic xanthochromia) from hemorrhage or the diapedesis of erythrocytes in severe tuberculous or suppurative meningitis, brain abscess, etc. It may also occur from venous stasis with transudation in cerebral tumors and especially tumors or other lesions producing compression of the spinal subarachnoid space. On the other hand, xanthochromia may be due to the presence of bile pigment in jaundice, carotinemia, or the administration of such drugs as neutral acriflavin.

3. A frequent cause is hemorrhage into the ventricles or subarachnoid space of the brain or spinal cord. When occurring without coagulation, it is suggestive of subdural hemorrhage. Under these conditions xanthochromia usually develops within 4 to 8 hours and increases in intensity while the number of erythrocytes decreases during the ensuing 4 to 8 days. It then decreases, unless there is a recurrence of bleeding, and usually disappears by the third week. On the other hand, the fluid may show a reddish color under these conditions due to spontaneous hemolysis. The xanthochromia of the newborn is ascribed to the presence of blood and especially after birth-injuries. Needless to state, the color is best detected after the fluid has been centrifuged. Fresh blood from a very recent hemorrhage or the accidental puncture of a vein, does not impart any color to the supernatant fluid, except at times, a slight reddish tinge from hemolysis.

4. The most pronounced examples of xanthochromia are observed in cerebrospinal fluids removed from *below* space constricting lesions of the spinal subarachnoid space and especially tumors of the spine or spinal cord. Under these circumstances the fluid is usually a clear yellow color with a great excess of protein and fibrinogen producing almost immediate coagulation which is commonly designated as "xanthochromia with massive coagulation" or the *syndrome of Froin*. Originally thought to be pathognomonic of spinal tumors, the syndrome is now known to be produced by any lesion compressing the spinal subarachnoid space with stagnation of the fluid and passive congestion as adhesions, localized pachymeningitis, etc.

5. Various observers have found the benzidine occult blood test of value in differentiating between the xanthochromia of the syndrome of Froin and that due to hemorrhage. In the former it is stated to give a negative reaction with the yellow color ascribed to venous stasis and transudation. In the latter it is stated to be

positive due to hemorrhage or inflammatory diapedesis of erythrocytes, for which the term "erythrochromia" has been proposed.

**Blood.**—In securing cerebrospinal fluid, a vein is sometimes punctured with the collection of pure blood and no fluid at all. It undergoes coagulation with the separation of serum in the usual manner.

However, blood evenly mixed with spinal fluid does not coagulate or but incompletely. This may occur when the lumen of the needle is partly in a vein and partly in the subarachnoid space. But if the physician believes that the needle is solely in the latter, the presence of blood indicates a recent hemorrhage communicating with the ventricles or the subarachnoid space of the brain or spinal cord. The absence of blood, however, does not exclude cerebral hemorrhage. Erythrocytes may be present in the cerebrospinal fluids of apparently healthy newborn infants as well as in those with birth-injuries.

**Reaction.**—The reaction of the normal cerebrospinal fluid is alkaline with a pH of 7.35 to 7.40 when determined immediately after collection. As shown by Levinson, it tends to become slightly more alkaline upon standing, due to the loss of  $\text{CO}_2$ . The alkaline reserve, as measured by the  $\text{CO}_2$  combining power, is also practically identical with that of blood plasma (55 to 75 volumes per cent).

The pH of the fluid is usually within normal in tuberculous meningitis but in the suppurative meningitides it is sometimes less alkaline (pH 7.2 to 7.5), the increase in acidity being due, in all probability, to an increase of lactic or other organic acids. Upon standing, the acidity shows much less tendency to decrease than in the case of normal fluids (Levinson). This is perhaps due either to an increased rate of glycolysis by bacteria, or to increased production of  $\text{CO}_2$  by the cells present in the fluid, balancing the loss of  $\text{CO}_2$  which occurs upon standing exposed to air. These changes, however, are of minor degree so that a determination of the pH of cerebrospinal fluids possesses little or no diagnostic value.

## TOTAL CELL COUNT

**Principles.**—1. Whenever possible, the total cell count should be made *immediately* after the collection of fluid while the cells are in suspension and before coagula have formed. If there is no excess of fibrinogen, so that coagulation does not occur, counts made some hours later or next day are fairly accurate, providing the fluid is well shaken to secure an even resuspension of cells. By collecting fluid in a tube carrying a minute amount of potassium oxalate, as described above, coagulation is prevented and counts made hours later compare quite closely with those made immediately after collection.

2. Needless to state, the counts must be made accurately when the total cells are but slightly increased, as in the case of clear or but faintly hazy fluids. When distinctly turbid or purulent, the counts are so high that slight errors make no difference from the diagnostic standpoint. When a slight or moderate increase of cells is of diagnostic importance, as in tuberculous meningitis, meningisms (serous meningitis), syphilis of the central nervous system, poliomyelitis, etc., accuracy is of paramount importance.

3. *Spinal fluids containing visible amounts of blood are unsuited for total cell counts* because of the presence of leukocytes resulting in counts that are too high.

Even traces of blood too small for macroscopic detection increase the total cells, which may result in diagnostic errors in diseases in which an increase of total cells is ordinarily between 20 to 100 per c.mm.

4. The total cells of normal cerebrospinal fluid obtained by lumbar or cisternal puncture vary from 0 to 8 per c.mm. in terms of undiluted fluid. In children the upper limit of normal is about 10 per c.mm. They are composed of small lymphocytes and for this reason are commonly regarded as hematogenous in origin. Slight differences, however, have been observed with fluids obtained from other loci; ventricular fluids, for example, may contain fewer cells than lumbar fluids.



FIG. 129.—FUCHS-ROSENTHAL RULING

5. An increase of cells is designated as *pleocytosis*. Under acceptable technical conditions a total cell count in adults of 9 to 12 per c.mm. is regarded as borderline; 13 to 30 represents a slight increase; 31 to 100 a moderate increase; 200 to 500 a marked increase, while 1000 or more represents a very marked increase.

**Procedure.**—1. The Levy counting chamber with the Fuchs-Rosenthal ruling (Fig. 129) is recommended. With the coverglass on it has a depth of 0.2 millimeter with a capacity of a trifle more than 3 cmm.

2. Draw diluting fluid to the mark 1 in the Thoma or Trenner leukocyte-counting pipets; draw spinal fluid to the mark 11.

#### DILUTING FLUID

Crystal violet .....	0.2 gm.
Glacial acetic acid.....	10.0 cc.
Water (distilled) .....	90.0 cc.
Filter. Should be crystal clear and free of artefacts.	

3. Shake well as in leukocyte counting and discard 2 or 3 drops.

4. Fill the Fuchs-Rosenthal chamber as in leukocyte counting and wait 5 minutes for the cells to settle.

5. Count all of the cells (erythrocytes are hemolyzed) in the entire ruled-off area and multiply by 0.35 to give the number of cells for each cubic millimeter of spinal fluid. The error incident to this calculation is practically balanced by the opposite error due to dilution.

6. If the Fuchs-Rosenthal chamber is not available, the ordinary leukocyte counting chamber may be used. In this case count the cells in the entire ruled off area (9 large squares, or 0.9 c.mm.); divide by 9 and multiply by 11. This calculation compensates for the dilution factor and gives the total cells per cubic millimeter of fluid.

#### DIFFERENTIAL CELL COUNTING AND CYTODIAGNOSIS

**Principles.**—1. A differential count is frequently of clinical value and known as *cytodiagnosis*. When fluids contain less than 300 cells per c.mm. they are, however, scarcely worthwhile because, aside from the difficulty of securing sufficient sediment for making the examination, experience has shown that under these conditions the cells are essentially small lymphocytes.

2. With higher total cell counts, however, differential counts are usually of additional clinical value. An excess of neutrophilic leukocytes (pus cells) is indicative of the acute purulent meningitides including aseptic meningitis. Endothelial cells from the meninges may be likewise present in meningisms or "serous meningitis" and are frequently classified as large lymphocytes. Elaborate classifications of the cells have been proposed, based upon sections of the fixed and hardened sediments, but possess no clinical value since differentiation into lymphocytes, neutrophils and endothelial cells serve all useful purposes. Tumor cells, however, are sometimes recognized by expert histopathologists and especially in cases of medulloblastoma.

**Procedure.**—1. Centrifuge fresh specimen of fluid. Pour off supernatant fluid and make thin smears of sediment on slides; or tease out coagula on slides. Dry in air.

2. Stain with Wright's stain in the same manner as blood smears.

3. Count and tabulate cells (lymphocytes, neutrophils and endothelial cells) and determine the number of each variety per 100 cells (see Fig. 124).

4. Normally, only lymphocytes and occasional endothelial cells are found. In acute suppurative meningitis due to the pneumococcus, meningococcus, streptococcus, etc., neutrophils predominate in the acute stage. In tuberculous meningitis, small lymphocytes predominate (usually). In acute anterior poliomyelitis, neutrophils early; later small lymphocytes (usually). In syphilis (paresis, tabes, etc.), small lymphocytes predominate. In meningisms (serous meningitis or acute meningeal congestion), endothelial cells predominate.

## QUALITATIVE TESTS FOR PROTEIN

**Principles.**—1. The protein content of the normal cerebrospinal fluid obtained by lumbar puncture is lower than that of any other normal body fluid with the exception of the aqueous humor of the eye, which it closely resembles in chemical composition, varying from 15 to 40 mg. (average 28 mg.) per 100 cc. Cisternal and ventricular fluids usually contain from 10 to 35 mg. per 100 cc. There is as yet no agreement concerning the type of protein present but it is apparently made up largely of albumin (23 mg. per 100 cc.), which is the most diffusible of the plasma proteins, with about 5 mg. of globulin per 100 cc., giving a ratio of about 6:1.

2. A large number of tests have been devised for the detection of an increase of protein in spinal fluid. Most of these have been for the detection of the globulins, but practically all react to some extent to serum albumin. Some were originally considered specific for syphilis of the central nervous system but none are pathognomonic for syphilis or any other disease; they merely detect an increase of protein (mostly globulins) which is always pathological unless the cerebrospinal fluid contains sufficient blood to yield positive reactions.

3. None of these tests, therefore, are applicable to cerebrospinal fluids containing macroscopic amounts of blood. Heavy bacterial contamination may likewise yield falsely positive reactions.

**Pandy's Test.**—1. Prepare the reagent by placing 100 cc. of pure phenol in a bottle and adding distilled water to 1000 cc. (melt crystals by standing bottle in hot water). Shake vigorously and place in incubator for several days. Carefully pipet off supernatant fluid or use it direct from the bottle without disturbing the layer of undissolved phenol.

2. Place about 1 cc. of the reagent in a small test tube. Add 1 drop of spinal fluid.

3. If there is an increased amount of protein, a bluish white ring or cloud is formed immediately. By this test normal spinal fluids often show a very faint trace of globulin which should not be mistaken for a positive reaction.

**Ross-Jones' Test.**—1. Place 1 cc. of saturated ammonium sulphate reagent in a test tube:

The *reagent* is prepared by placing 85 grams of Merck's purified and neutral ammonium sulphate and 100 cc. of distilled water in an Erlenmeyer flask; heat to boiling until all of the salt is dissolved. Cool slowly and filter.

2. Overlay with 1 cc. of the fluid to be tested.

3. The appearance of a turbid ring at the point of contact—in a few seconds—indicates positive reaction. Normally a ring may appear within 5 minutes to 3 hours.

**Nonne-Apelt's Test.**—**PHASE 1 FOR GLOBULIN.**—In a small test tube place 2 cc. of spinal fluid and 2 cc. of the ammonium sulphate reagent (see above). Allow to stand 3 minutes. Compare with spinal fluid. A normal fluid gives no reaction or but a faint opalescence.

*Phase 2 for Albumin.*—Filter contents of tube just prepared; acidulate with acetic acid and boil. A normal fluid remains clear or but faintly opalescent.

**Tryptophan Test for Tuberculous Meningitis.**—1. This simple test is said to be of helpful diagnostic aid.

2. Place 2 or 3 cc. of spinal fluid in a small test tube.

3. Add 0.5 to 0.8 cc. of concentrated hydrochloric acid and 2 or 3 drops of 2 per cent formaldehyde (1 cc. of formalin and 19 cc. distilled water).

4. Shake the tube and allow to stand for 5 minutes.

5. Carefully overlay with 2 cc. of 0.6 per cent solution of sodium nitrate.

6. Allow to stand for 2 or 3 minutes.

7. In tuberculous meningitis a violet ring may develop at the line of contact (positive reaction). A negative reaction is shown by a brown ring, or the absence of any colored ring.

**Levinson Test for Tuberculous Meningitis.**—This test is based upon the principle that a characteristic ratio may be obtained between the alkaloidal precipitate formed by sulphosalicylic acid and the metallic precipitate formed by mercuric chloride in tuberculous meningitis. While positive reactions are indicative of this infection, they are not specific.

1. Into each of two small test tubes of uniform length and width place 1 cc. of cerebrospinal fluid.

2. To one add 1 cc. of a 3 per cent solution of sulphosalicylic acid (C.P.) in water and to the second 1 cc. of a 1 per cent solution of mercuric chloride (C.P.) in water.

3. Allow to stand at room temperature for 24 hours when the sediments are measured and compared.

4. Under *normal* conditions the sediment in both tubes is very slight. In all *suppurative meningitides*, the height of the sediment in the sulphosalicylic acid tube is very heavy, often being three times the size of the sediment occurring with mercuric chloride. In *tuberculous meningitis* (rarely in other conditions) the opposite occurs, the precipitation with mercuric chloride being usually 3 times as high as that obtained with sulphosalicylic acid.

The 2 precipitates are of a different character; that of the acid is heavy and compact and starts to form immediately, while that of the chloride is light, feathery, and forms slowly. Sometimes the precipitate does not come down into a compact sediment as small floccules may adhere to the walls of the test tube. Under these conditions it is advisable to gently shake the tubes 2 or 3 hours before making the final readings. For diagnostic purposes it is not the amount of protein thrown down in the 2 precipitates, but the relative height of the sediments in millimeters in the two tubes.

If no precipitate forms, use a 2 per cent mercuric chloride solution and a 6 per cent sulphosalicylic acid solution.

### QUANTITATIVE TESTS FOR TOTAL PROTEIN

**Method of Kingsbury, Clark, Williams and Post.**—The technic of this method is the same as for the quantitative determination of albumin in the urine described on page 132.

1. Place 1 cc. of spinal fluid in a test tube of the same size as the standards.
2. Add 3 cc. of 3 per cent solution of sulfosalicylic acid reagent and mix thoroughly.
3. Allow to stand for 5 minutes and compare the degree of turbidity with that of the permanent standard tubes. A Clark lamp aids the reading.

4. The normal total protein by this method is usually about 30 mg. per 100 cc.

**Method of Johnston and Gibson.**—1. Place 2 cc. of spinal fluid in a 15 cc. centrifuge tube. Add 3 cc. of distilled water and 1 cc. of 20 per cent solution of trichloroacetic acid.

2. Mix by inversion, wait a few minutes, and centrifuge.
3. Pour off and discard the supernatant fluid.
4. Add 0.25 cc. of a 10 per cent solution of sodium hydroxide to the precipitate and heat in a boiling water bath for 10 minutes.
5. Add 3.75 cc. of distilled water, 0.5 cc. of phenol reagent (Folin-Ciocalteu) and 1.5 cc. of a saturated solution of sodium carbonate.
6. Mix by inversion, allow 10 minutes for color to develop and compare with the nearest standard in the colorimeter with the standard set at 10 mm.

7. Three standards should be prepared as follows: *0.5 standard:* To 0.5 cc. of standard tyrosine solution (20 mg. dissolved in 100 cc. of N/10 hydrochloric acid) add 7.5 cc. of distilled water, 1 cc. of Folin-Ciocalteu color reagent and 3 cc. of a saturated solution of sodium carbonate. With this standard  $\frac{285}{R} =$  mg. per cent of protein.

*1.0 standard:* 1 cc. of the standard tyrosine solution, dilute to 8 cc. and treat as above. With this standard  $\frac{570}{R} =$  mgm. per cent of protein.

*2.0 standard:* 2 cc. of the standard tyrosine solution, dilute to 8 cc. and treat as above. With this standard  $\frac{1140}{R} =$  mg. per cent of protein.

### QUALITATIVE TEST FOR GLUCOSE

**Principles.**—1. It is now generally agreed that the glucose of the cerebrospinal fluid is derived from that of the blood. Consequently, it varies not only (*a*) according to the blood sugar concentration, but also according to (*b*) the permeability of the choroid plexus and possibly of the capillaries surrounded by prolongations of the sub-arachnoid space, as well as to (*c*) the rate of glycolysis in the fluid. Furthermore, it is possible that the cells of the choroid plexus may utilize some of the sugar.

2. *Determinations of cerebrospinal fluid sugar are best made with fluid collected after a period of fasting, preferably over night, with a blood sugar estimation at the same time.*

3. In view of the variable factors concerned in cerebrospinal fluid glucose the normal cannot be stated accurately, but is generally regarded as varying from 50 to 75 mg. per 100 cc. in adults with slightly larger amounts in cisternal and ventricular fluids. In children up to 10 years of age the normal has been found somewhat higher, varying from 70 to 90 mg. per 100 cc. Cerebrospinal fluid also contains nonglucose reducing substances which disappear on hydrolysis, amounting to about 4.0 mg. per 100 cc. or about 10 per cent of the total. The ratio of fluid to blood glucose likewise cannot be accurately stated but, in normal individuals, under fasting conditions, the cerebrospinal fluid glucose is thought to be approximately 45 to 70 per cent of the blood glucose.

4. If the fluid contains an increase of protein and an absence of sugar, the color of the reagent may be changed to a deep purplish-violet or pinkish-violet (the biuret reaction with copper).

5. Since blood sugar will give positive reactions, spinal fluids containing macroscopic amounts of blood are unfit for testing.

6. An increase of cerebrospinal fluid glucose is designated as *hyperglycorachia* and a decrease as *hypoglycorachia*.

**Procedure.**—1. In a test tube place 0.5 cc. of Benedict's *qualitative* reagent and add 4.5 cc. of distilled water.

2. Add 0.5 cc. of cerebrospinal fluid.

3. Boil for 1 to 2 minutes and allow to cool.

4. A change of color to turbid greenish-yellow is a normal reaction for the normal sugar of spinal fluid. No color change shows an absence of sugar and is pathological. An excess of protein may give a biuret reaction as mentioned above.

### QUANTITATIVE TESTS FOR GLUCOSE

**Folin-Wu Test.**—This test is conducted according to the method of Folin and Wu for blood glucose as follows:

1. Dilute 2 cc. of cerebrospinal fluid with 8 cc. of distilled water and use in place of the protein-free filtrate.

2. Since 2 cc. of the cerebrospinal fluid represents 0.4 cc. of undiluted fluid, calculate as follows with *D* representing the number of milligrams of glucose in the standard:

$$\frac{\text{Reading of the Standard}}{\text{Reading of the Unknown}} \times D \times 250 = \text{mg. of glucose in 100 cc. of cerebrospinal fluid.}$$

**Lytle and Hearn Test.**—The reagents required are the same as those used in the Folin-Wu method for the precipitation of blood proteins and determination of blood glucose.

1. Four volumes of spinal fluid are added to 14 volumes of distilled water and to this mixture 1 volume of 10 per cent sodium tungstate is added, followed by 1 volume of N/1.5 sulfuric acid. Shake and allow to stand for 10 minutes, then filter. The glucose determination is carried out on 2 cc. of this filtrate in exactly the same manner as in determining blood glucose.

2. Where the lower standard has been used, the reading of the standard, usually 20 mm., multiplied by 50 and divided by the reading of the unknown, equals milligrams of glucose per 100 cc. of spinal fluid. When the higher standard is used, substitute 100 for the 50 above.

### QUANTITATIVE TEST FOR CHLORIDES

**Principles.**—1. The chlorides of the cerebrospinal fluid are expressed in terms of sodium chloride. The normal varies from 720 to 750 mg. per 100 cc., being the same for lumbar, cisternal and ventricular fluids. Values in infants exhibit slightly more variation, ranging from 650 to 720 mg. per 100 cc. Normal cerebrospinal fluid, therefore, contains more chloride than the normal blood plasma (570 to 620 mg. per 100 cc.) from which it is derived through the free permeability of the choroid plexus.

2. In the absence of meningitis the chloride of the fluid naturally varies to some extent with that of the plasma. For example, it may be increased in some cases of nephritis with hyperchloremia while decreased in states of hypochloremia, as occurs in lobar pneumonia, pyloric obstruction, etc. Consequently, the plasma chloride concentration must be considered in interpreting reduced amounts of chloride in the cerebrospinal fluid, particularly in acute infections, like pneumonia, in which the development of symptoms of meningism may arouse a suspicion of meningitis.

3. The chloride estimation is valuable in diagnosis when considered in conjunction with the quantity of glucose present. Chloride values below 600 milligrams are infrequent except in tuberculous meningitis, while values between 630 and 680 milligrams per 100 cc. are commonly found in acute purulent meningitis.

4. Since the presence of blood in spinal fluid may alter the chloride content, quantitative determinations cannot be done on fluids containing macroscopic amounts of blood.

5. Qualitative tests are useless.

**Procedure.**—Same as for the qualitative estimation of plasma chlorides; 1 cc. of spinal fluid is diluted with 9 cc. of water for the determination.

### LANGE COLLOIDAL GOLD TEST

**Principles.**—1. Normal cerebrospinal fluid does not visibly precipitate gold in colloidal suspension in properly prepared Lange's reagent.

2. In syphilis of the central nervous system precipitation may occur, varying from a slight precipitation indicated by a change of color from orange-red to reddish-blue to purple-blue to pale blue to complete decolorization. By means of these color changes, curves of precipitation may be plotted which have a great deal of diagnostic



value. Less characteristic precipitations (color changes) occur in tuberculous and acute suppurative meningitis and may also occur in acute anterior poliomyelitis and other diseases of the central nervous system.

3. The exact chemical and immunological nature of the precipitating substance in spinal fluid is unknown.

4. While the test is simple and easily set up and read, the reagent is difficult to prepare and, unless just right with the proper color and neither too sensitive nor too resistant (protected), may readily yield falsely positive or falsely negative reactions. The colloidal gold reaction is, therefore, a valuable diagnostic test but greatly subject to technical errors.

**Preparation of Reagent.**—In preparing the reagent the glassware should be chemically clean and should be rinsed inside and out with double-distilled water before using. Pyrex glass is not essential except in the flask used for boiling. Quicker heating without wire gauze may thus be attained and is important. It is also better to store the completed reagent in pyrex but not absolutely necessary.

1. Prepare a 1 per cent solution of gold chloride as follows: Place an ampule of Merck's gold chloride (15 grains) in warm water and remove the label and all paste. Rinse thoroughly in double-distilled water before breaking. Take care that none of the chloride is lost in breaking. Place the halves of the ampulae, with their contents, in beaker containing 97.2 cc. of double-distilled water and stir thoroughly to insure complete and even solution of the chloride.

2. Prepare a 1 per cent solution of sodium citrate, C.P., in double-distilled water by dissolving 1 gm. (accurately weighed) in 100 cc.

3. Pipet 10 cc. of the gold chloride solution into 950 cc. of double-distilled water in a 2000 cc. Erlenmeyer flask.

4. Heat, as rapidly as possible, to between 90° and 95° C. (*no higher and no lower*).

5. Remove thermometer from flask as soon as it records 92° C.

6. Without removing from the flame, add 50 cc. of the citrate solution.

7. As soon as the solution comes to a boil again add quickly 0.77 cc. of hydrogen peroxide. When drawing up the peroxide into the pipet, take care to exclude the many small bubbles that form in a freshly opened bottle. The hydrogen peroxide should be C.P. (10 volumes, 3 per cent). It should be a freshly opened bottle. *Never* use one that has been standing, uncorked, at room temperature for any length of time.

8. Remove from flame, cool, and store in a dark place at room temperature. Cork tightly with a tinfoil-covered stopper.

9. *Do not shake the flask during procedure.*

10. When the solution begins to re-heat after the addition of the citrate, a slight bluish color begins to appear which gradually changes to the standard color during 3 minutes of boiling. No further change takes place thereafter. The addition of the peroxide, with its immediate color change, gives the required sensitivity. The solution must not be boiled after the peroxide is added. Titration is not necessary if the final color change (brilliant salmon or orange-red color with no bluish tinge) is right.

The method of *Miller, Brush, Hammers and Felton* is as follows:

1. All glassware should be boiled or well washed with ivory soap and water, rinsed with running tap water, soaked for 30 minutes or longer in hot bichromate cleaning fluid and *immediately before use* rinsed thoroughly in running tap water,

distilled water, and lastly in triply distilled water. The thermometer should be carefully washed and rinsed with distilled water just before use.

2. The water used for preparing the reagents and the colloidal gold reagent should be triply distilled with avoidance of rubber connections in the still. It is recommended that the third distillation be done just before preparing the reagent.

3. The following reagents are required: (a) A 1 per cent solution of Merck's yellow crystals of gold chloride in triple distilled water; (b) a 2 per cent solution of Merck's blue label potassium carbonate in triple distilled water; (c) a 1 per cent solution of Merck's blue label oxalic acid in triple distilled water; (d) Merck's highest purity formalin (40 per cent formaldehyde in distilled water).

4. Place 1000 cc. of triply distilled water and a thermometer in a prepared beaker and heat slowly with a triple Bunsen burner.

5. At 60° C. add 10 cc. of the 1 per cent gold solution and 7 cc. of the freshly prepared 2 per cent potassium carbonate solution, using the thermometer as a stirring rod.

6. At 80° C. add slowly 10 drops of the 1 per cent solution of oxalic acid while briskly stirring.

7. At 90° C. turn out the flame and add the formalin drop by drop while constantly stirring until the *first pink color appears* (approximately 5 cc. ordinarily required).

8. The reagent should gradually assume a brilliant orange-red color without bluish tint.

9. A *shorter method* usually yielding satisfactory results is as follows: (a) Into a beaker cleaned as outlined above, place 500 cc. of singly distilled water. (b) Place beaker on asbestos-filled wire gauze and heat over Bunsen burner. Stir the solution to keep the heat distributed while heating and gradually add 5 cc. of 1 per cent solution of gold chloride. (c) As soon as the solution begins to boil, turn off the flame and add 5 cc. of 2 per cent potassium carbonate solution and 0.15 to 0.2 cc. of 0.05 per cent solution of tannic acid in distilled water. (d) Allow solution to come to boil and add 5 cc. of a 1 per cent solution of oxalic acid while stirring. Remove from flame. (e) The solution should develop a salmon or orange-red color and be perfectly clear and sparkling.

The *method of Borowskaja* is simple and frequently satisfactory. Add 1 cc. of 1 per cent solution of gold chloride (Merck's "blue label") to 95 cc. of distilled water. Heat to 90° C., and add 5 cc. of 1 per cent solution of sodium citrate (Merck's "blue label"). Boil for 1 to 3 minutes. Set aside to cool. Use scrupulously clean pyrex glassware.

By whatever method of preparation, the reagent should be crystal clear and of a brilliant salmon or orange-red color with no trace of blue.

Furthermore, it must not be too sensitive (acid) or insufficiently sensitive (alkaline). For these reasons it should be allowed to stand at least 2 hours after preparation and then tested with a known positive spinal fluid (paretic preferred) and a known negative or normal fluid. The former should give a typical Zone I or paretic curve like 5554321000 and the latter a negative reaction, like 0000000000 or 1000000000; 5 cc. of the reagent must be capable of being completely decolorized in 1 hour when 1.7 cc. of a 1 per cent solution of sodium chloride has been added.

**Procedure.**—1. Place 11 *chemically clean* test tubes in a rack.

2. Into the first tube place 1.8 cc. of 0.4 per cent *sodium chloride solution* and 1 cc. in each of the remaining 10 tubes.

3. Add 0.2 cc. of spinal fluid to the first tube and thoroughly mix.

4. Remove 1 cc. from the first tube and place in the second tube; mix thoroughly and remove 1 cc. and place in the third tube; continue until the tenth tube is reached and then discard 1 cc. from this tube. The eleventh tube is used as a control.

5. Add to each tube 5 cc. of colloidal gold reagent.

6. Mix thoroughly and set aside for 24 hours, at the end of which time the readings are made.

7. Readings: Each tube is examined and the reaction recorded, using the numbers 0 to 5.

0 = unchanged as compared with the control

1 = reddish-blue

2 = lilac or purple

3 = blue

4 = almost colorless (trace of blue)

5 = colorless

8. The readings are recorded in the order in which the tubes stand (Fig. 130).  
For example:

5, 5, 5, 5, 4, 3, 1, 0, 0, 0 = curve in the paretic zone (Zone I)  
(Plate VI)

1, 1, 2, 3, 2, 1, 0, 0, 0, 0 = curve in the luetic zone (Zone II)  
(Plate VII)

0, 0, 0, 1, 2, 3, 4, 5, 2, 0 = curve in the meningitic zone

0, 0, 0, 0, 0, 0, 0, 0, 0, 0 = negative

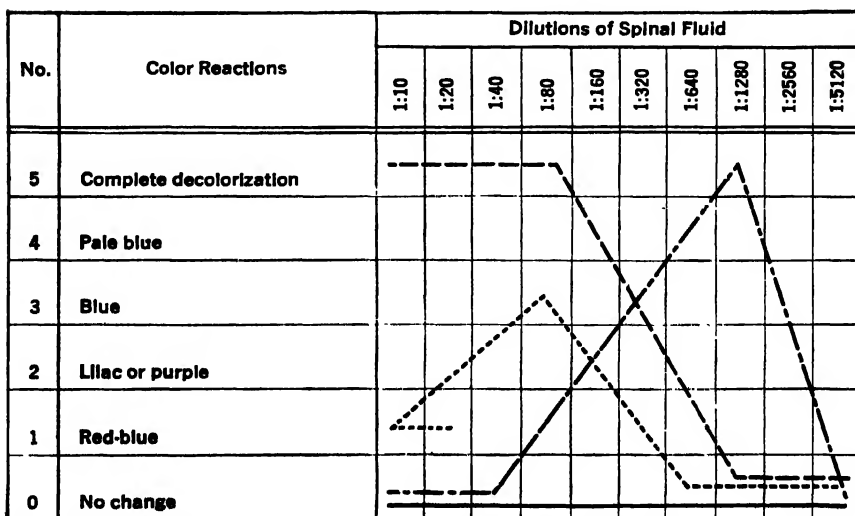
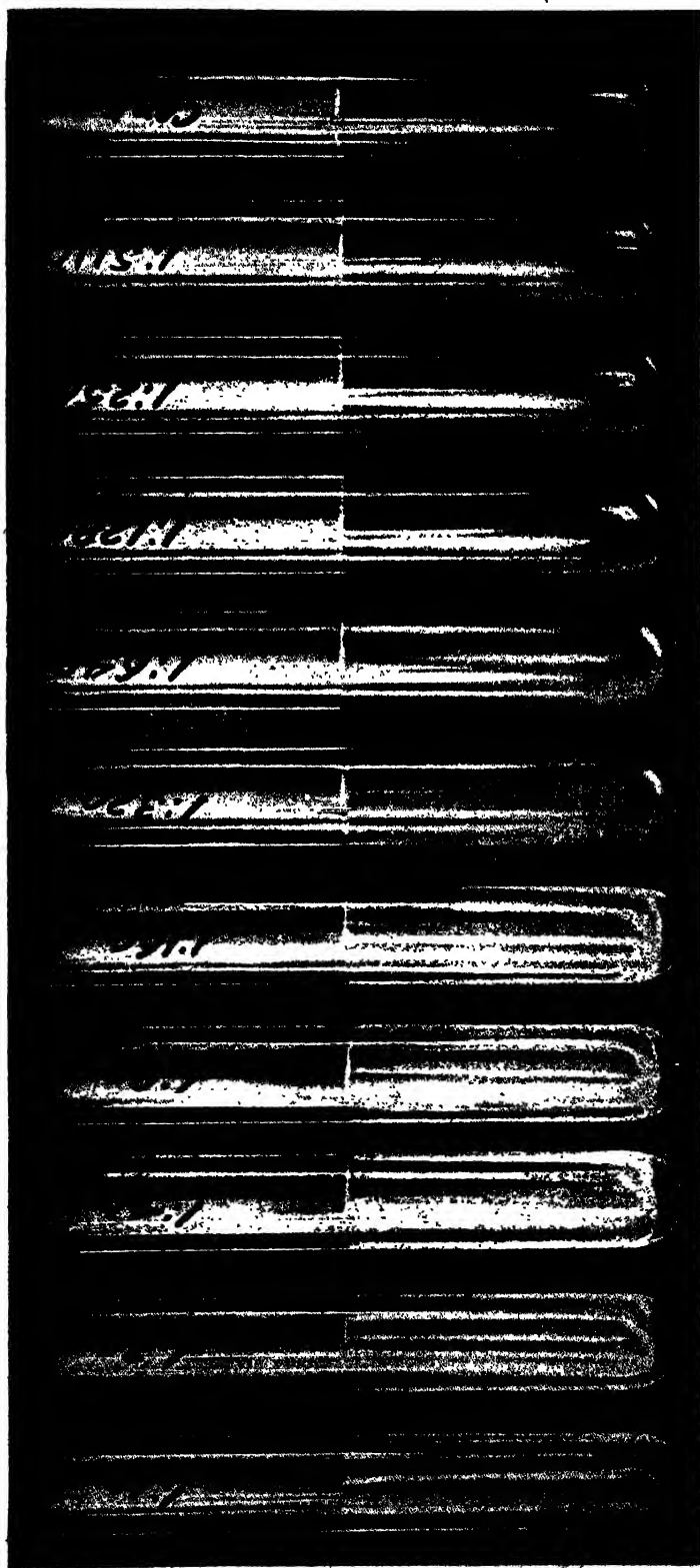


FIG. 130.—THE FOUR COMMON TYPES OF COLLOIDAL GOLD REACTIONS

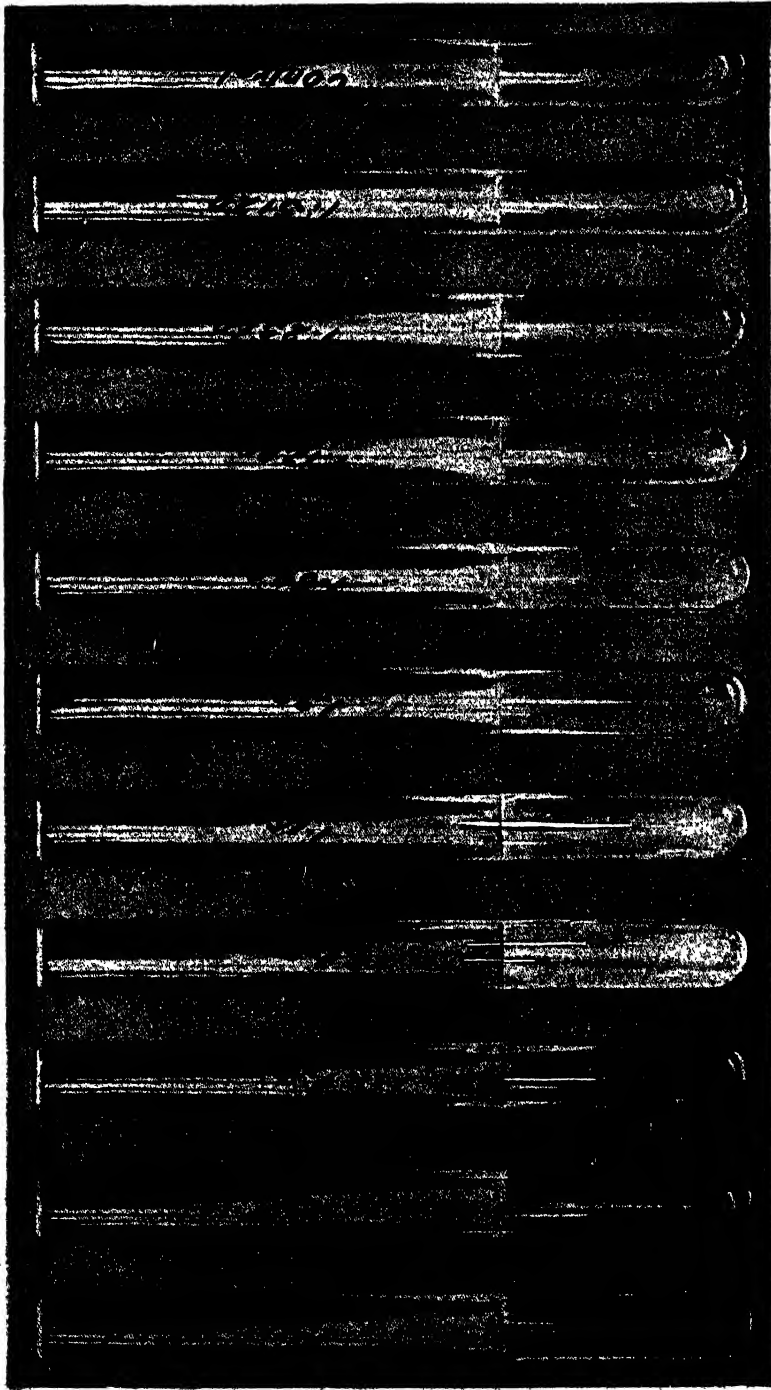
----- Paretic curve or Zone I  
 . . . . . Luetic curve or Zone II  
 -.-.-.- Meningitic curve  
 ----- Negative

PLATE VI



COLLOIDAL GOLD REACTION (PARETIC OR ZONE I CURVE)  
(From Kolmer. *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

PLATE VII



THE COLLOIDAL GOLD TEST WITH THE CEREBROSPINAL FLUID OF TABES DORSALIS, SHOWING A "LUETIC ZONE" REACTION

(0125522000)

(From Kolmer, in Frazier, *Surgery of the Spine and Spinal Cord*, D. Appleton-Century Company, New York.)

# CEREBROSPINAL FLUID EXAMINATION

Name:  
Clinical Diagnosis:

Age:

Physician:

Date:

Pressure (Millimeters of Mercury)	Amount Removed (C.C.)	Physical Properties	Cells per C. Mm.	Differential Cell Count			Protein Tests		Complement- fixation Reaction
				Lymph.	Polys.	Endothel.	Qualita- live	Quantitative	
Before.....							Pandy	0.2 0.1 0.05	Blood
After.....									Spinal Fluid

## COLLOIDAL GOLD AND MASTIC REACTIONS\*

Regis- try No.	Color Reactions	Dilutions of Spinal Fluid										Remarks
		1 1:10	2 1:20	3 1:40	4 1:80	5 1:160	6 1:320	7 1:640	8 1:1280	9 1:2560	10 1:5120	
5	Colorless.....											
4	Pale blue.....											
3	Blue.....											
2	Lilac or purple.....											
1	Red-blue.....											
0	Red-unchanged.....											

\* — gold curve; ..... mastic curve.

Serum:

Dextrose:

Quantitative Kolmer Reaction:

Serum 0.2 c.c.

Serum 0.1 c.c.

Serum 0.05 c.c.

Serum 0.025 c.c.

Serum 0.005 c.c.

Bacteriological Examination:

Culture:

Animal Inoculation:

Quantitative Chloride:

Quantitative Kolmer Reaction:

Spinal Fluid 0.5 c.c.

Spinal Fluid 0.25 c.c.

Spinal Fluid 0.125 c.c.

Spinal Fluid 0.0625 c.c.

Spinal Fluid 0.03125 c.c.

Examined by:

FIG. 131.—THE KOLMER CHART FOR SPINAL FLUID EXAMINATIONS  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

The chart shown in Figure 131 is recommended for reporting the results of the colloidal gold and other spinal fluid examinations.

9. The *method of Boerner and Lukens* is equally satisfactory and more economical of cerebrospinal fluid and reagent:

(a) Place 1.8 cc. of 0.4 per cent sodium chloride solution in the first tube and 0.5 cc. in each of the remaining 10 tubes.

(b) Add 0.2 cc. of spinal fluid to the first tube. Mix, discard 1 cc. and transfer 0.5 cc. to the second tube.

(c) Mix and transfer 0.5 cc. to the third tube, and so on to the next to the last tube, from which 0.5 cc. is discarded after mixing.

(d) Add 2.5 cc. of reagent to all tubes and complete the test as described above. The readings are made in the same manner.

### CUTTING'S COLLOIDAL MASTIC TEST

**Principles.**—1. This test depends upon the precipitation of mastic in colloidal suspension as determined by a clarification of the reagent and the production of precipitates.

2. It is highly probable that the substance in spinal fluid producing the reaction is the same as that producing the colloidal gold reaction, although its nature is unknown.

3. The reagent is much simpler and easier to prepare, although the reactions are less sensitive than the colloidal gold reaction.

4. The reaction, while less sensitive, is also less subject to technical errors.

**Preparation of Reagents.**—1. A stock solution of mastic is prepared by completely dissolving 10 grams of U.S.P. gum mastic in 100 cc. of absolute alcohol. Filter.

2. For use dilute 2 cc. with 18 cc. of absolute alcohol, mix well, and pour rapidly into 80 cc. of freshly distilled water.

3. Prepare a 1.25 per cent solution of C.P. sodium chloride in distilled water, and to each 99 cc. add 1 cc. of a 0.5 per cent solution of potassium carbonate in distilled water (alkaline-saline solution).



FIG. 132.—COLLOIDAL MASTIC REACTIONS

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

**Procedure.**—1. Arrange 6 small test tubes in rack.

2. Place 1.5 cc. of alkaline-saline solution in the first tube and 1 cc. in each of the remaining 5 tubes.

3. Add 0.5 cc. of spinal fluid to the first tube, mix thoroughly and transfer 1 cc. to the second tube.

4. Transfer 1 cc. from the second tube to the third and so on until the fifth tube, from which 1 cc. is discarded. The sixth tube is used as a control.

5. To each tube add 1 cc. of mastic reagent, mix well and allow to remain at room temperature for 12 to 24 hours; or in the incubator for 6 to 12 hours.

6. A positive reaction is indicated by the formation of a heavy precipitate which settles, leaving the supernatant fluid clear (Fig. 132).

### COLLOIDAL BENZOIN TEST

This test, devised by Guillain, Laroche, and Lechelle, is similar in many respects to the mastic test. It is not specific for neurosyphilis, but gives practically the same results as the more complicated colloidal gold test.

**Preparation of Reagents.**—1. *Benzoin Solution.*—Sumatra benzoin resin, 1 gram; absolute alcohol, 10 cc. After 48 hours filter off the clear supernatant fluid. Keep in a tightly stoppered bottle. This is a stock solution from which the colloidal solution which is used in the test is freshly prepared each day as follows:

Add 0.3 cc. of the stock benzoin solution, drop by drop with constant shaking, to 20 cc. of doubly distilled water. Heat to 35° C. in a water bath with constant shaking.

2. *Salt Solution.*—Prepare 0.01 per cent sodium chloride in doubly distilled water.

**Procedure.**—1. Set up in a rack 16 small test tubes (75 by 10 millimeters, or 85 by 13 millimeters).

2. In the first tube place 0.25 cc. of salt solution; in the second tube, 0.5 cc.; in the third, 1.5 cc., and in each of the remaining tubes 1 cc.

3. Next add cerebrospinal fluid: 0.75 cc. to the first tube; 0.5 cc. to the second and third tubes. From the third tube 1 cc. of the thoroughly mixed dilution of spinal fluid is transferred to the fourth tube, and so on, until the fifteenth tube is reached from which, after mixing, 1 cc. is discarded. The sixteenth tube is used for control. The dilutions thus range from 3:4 in the first tube to 1:16,384 in the fifteenth tube.

4. Finally, add 1 cc. of the benzoin suspension to each tube and mix by shaking. The tubes are allowed to stand for from 18 to 24 hours.

5. The reaction will vary from no change in the mixture to complete precipitation of the benzoin, with absolute clearing of the supernatant fluid. The degree of reaction in each tube is reported: 0, no precipitation; 1, slight precipitation, with partial clearing; 2, more than half precipitated, fluid still cloudy; 3, complete precipitation, water-clear fluid. A curve may be plotted, or the figures representing the degree of reaction may be set down for each tube. Precipitation in the first 6 tubes indicates cerebral involvement, the first, or parietic zone; precipitation beginning with the seventh tube indicates involvement of the meninges, or spinal cord, the second, or meningeal zone. The test is not as sensitive as in the Lange colloidal gold method, and is not as definite in its reaction in multiple sclerosis.



**KOLMER COMPLEMENT FIXATION TEST**

The method of conducting this test is described on pages 674 to 698.

**BACTERIOLOGICAL EXAMINATION**

1. As soon as spinal fluid is received in the laboratory, it should be cultured on blood agar or some other suitable medium before any other examinations are made, in order to avoid contamination.
2. If very cloudy, direct smears may be made on slides. If opalescent, a portion should be centrifuged and smears prepared of the sediment.
3. Smears should be stained by methylene blue and Gram's method.
4. Methods for the detection and identification of meningococci, pneumococci, streptococci, influenza and tubercle bacilli and other organisms are described on pages 413 to 414.

**CEREBROSPINAL FLUID IN DISEASE**

The accompanying charts briefly summarize the more important changes in those diseases in which cerebrospinal fluid examinations have proved of value in diagnosis.

**COMPARISON OF NORMAL CEREBROSPINAL FLUID AND BLOOD PLASMA**

(MERRITT and FREMONT-SMITH)

	Cerebrospinal Fluid		Plasma
	Range	Average	Average
Specific gravity .....	1.006 to 1.009	1.0075	1.025
Total solids * .....	0.83 to 1.77	1.00	8.7
Water content * .....	98.23 to 99.17	99.00	91.3
Freezing point —° C....	—0.534 to 0.603	—0.570	—0.570
Chloride † .....	424.0 to 454.0	440.0	360.0
Chloride (as NaCl) † ....	700.0 to 750.0	726.0	594.0
Bicarbonate ‡ .....	.....	21.0	23.0
Phosphorus † .....	1.2 to 2.1	1.5	4.0
Lactic acid † .....	10.0 to 20.0	15.0	15.0
Sodium † .....	301.0 to 343.0	324.0	316.0
Potassium † .....	11.0 to 15.0	13.0	19.0
Calcium † .....	4.5 to 5.5	5.0	10.0
Magnesium † .....	1.0 to 3.5	3.0	2.0
Total base ‡ .....	.....	155.0	162.0
Protein † .....	15.0 to 45.0	28.0	7000.0
Albumin .....	.....	23.0	4430.0
Globulin .....	.....	5.0	2270.0
Fibrinogen .....	.....	.....	300.0
Nonprotein nitrogen † ....	11.0 to 38.0	19.0	27.0
Urea † .....	8.0 to 28.0	14.0	14.0
Creatinine † .....	0.5 to 1.9	1.1	1.6
Amino-acid † .....	1.2 to 2.0	1.6	5.0
Uric acid † .....	0.4 to 2.8	1.7	4.7
Cholesterol † .....	0.06 to 0.22	0.14	160.0
Reducing substances † ....	50.0 to 80.0	65.0	98.0
Glucose .....	.....	61.0	92.0
Nonglucose .....	.....	4.0	6.0

\* Grams per 100 cc.

† Milligrams per 100 cc.

‡ Millimols per liter.

# SUMMARY OF THE USUAL CEREBROSPINAL FLUID CHANGES IN DISEASE

Disease	Pressure*	Character	Coagulation	Cytology†	Qualitative Globulin	Qualitative Albumin	Quantitative Protein (Mg. per 100 c.c.)	Quantitative Sugar (Mg. per 100 c.c.)	Chlorides (Mg. per 100 c.c.)	Bacteria	Wassermann Reaction	Colloidal Gold Reaction
Normal	100 to 200	Clear and colorless	Absent	0 to 8 Lymphocytes	-	-	15 to 40	+	720 to 750	None	Negative	Negative
Serous meningitis (meningismus)	Increased	Normal	Absent	Normal or slight increase of endothelial	-	-	Normal or slight increase	+	Normal	None	Negative	Negative
Anterior poliomyelitis	Increased	Normal or opalescent	Fibrin web (occasionally)	0 to 2000 Early; Polymorphonuclears Later Lymphocytes	+	++	40 to 500 Slight increase in 30 per cent	++	Normal	None	Negative	Meningitic or Zone II curve‡
Purulent meningitis	Marked increase	Slightly cloudy to thick pus, often xanthochromic	Thick coagulum	100 to 5000 Polymorphonuclears	+++	++++	Marked increase up to 5000	± to -	Normal or slight increase	Present	Negative	Meningitic curve
Chronic basilar meningitis	Normal or increased	Normal or opalescent; often xanthochromic	Coagulum	10 to 1000 Polymorphonuclears	+++	++++	100 to 1000	± to +	Normal	None or small amount of cocci	Negative	Meningitic curve
Tuberculous meningitis	Usually increased‡	Usually clear	Fibrin web	80 to 1000 Lymphocytes (occasionally polymorphonuclears predominate)	+++ to ++++	+++ to ++++	100 to 1000	± to -	500 to 700	Tubercle bacillus	Negative	Meningitic curve
Epidemic encephalitis	Normal or increased	Normal, bloody or xanthochromic	Fibrin clot occasionally	10 to 200 Lymphocytes	± to +	++ to ++	30 to 200	++	Normal	None	Negative	Negative or Zone II curve
Brain tumor	Variable	Normal or xanthochromic	Absent	10 to 80 Lymphocytes	± to +	++ to ++	20 to 200	++ to ++	Normal	None	Negative	Negative
Intracranial tumor (compression syndrome)	Variable	Normal or xanthochromic	Massive coagulation	Normal to 50 Lymphocytes	++ to ++++	++ to ++++	60 to 1000	+	Normal or slight increase	None	Negative	Negative
Syphilis (primary and secondary stages)	Normal	Normal	Absent	8 to 28 Lymphocytes	± to +	++ to ++	20 to 60	+	Normal	None	Variable	Negative or basile (Zone II) curve rarely
Syphilis (meningovascular)	Normal or slight increase	Normal	Absent	2 to 1000 Lymphocytes 60 to 75 per cent	++ to ++	++ to ++++	30 to 150	+	Normal or reduced	None	Positive	Luetic (Zone II) curve
Syphilis (tabes dorsalis)	Normal	Normal	Absent	10 to 75 Lymphocytes	± to +	++ to ++	30 to 60	+	Normal or reduced	None	Positive in 70 per cent	Luetic (Zone II) curve
Syphilis (gummata)	Normal or slight increase	Normal	Usually small coagula	30 to 200 Lymphocytes	++ to ++++	++ to ++++	50 to 100	+	Normal or reduced	None	Positive in 100 per cent	Parvitic (Zone I) curve
Multiple sclerosis	Normal or slight increase	Normal	Absent	0 to 40 Lymphocytes	± to ++	++ to ++	20 to 80	+	Normal	None	Negative	50 per cent negative. Luetic (Zone II) or Parvitic (Zone I) curve may occur

\* Pressure is given in milliliters of water. To convert to mercury, divide by 13.

† Only the predominating cells are mentioned.

‡ The Zone II curve often occurs during the early stages of acute poliomyelitis.

§ If blocking of the foramina in the cisternae occurs or the protein becomes plastic the pressure may be decreased.

## METHODS FOR THE EXAMINATION OF THE BLOOD AND URINE FOR HORMONES

**Principles.**—1. Hormones are chemical substances secreted by the ductless glands which, when carried to other glands and tissues of the body by the blood, stimulate or inhibit their functional activities. They are known as hormones, a name derived from the Greek word meaning to excite, because they are predominantly excitatory in nature. The ductless glands are nine in number comprising the pituitary, pineal, thyroid, parathyroids, thymus, the islands of Langerhans of the pancreas, adrenals, testicles and ovaries. Apparently, however, other organs may likewise secrete hormones concerned in their own physiologic activities or those of other organs. Thus the placenta apparently produces a hormone which, acting through the mediation of the ovaries, ensures its own physiological integrity although it may be secreted elsewhere and merely stored in the placenta. Hormones are also secreted by the stomach and duodenum and possibly by the liver, spleen and other organs.

2. Unfortunately, laboratory methods for the determination of the hormones occurring in the blood, or excreted in the urine, are only available in the case of the anterior-pituitary-like hormone, the gonadotropic hormones produced by the anterior lobe of the pituitary gland, the hormones of the ovary and the hormones or androgens of the testicles. But, various other laboratory methods are available for the detection of an increased or decreased production of some of the hormones as, for example, the basal metabolism test in relation to the thyrotropic hormone of the pituitary gland as well as of the thyroid gland hormone itself, blood glucose and glucose tolerance tests in relation to the ketogenic and adrenotropic hormones of the pituitary gland and of adrenalin of the adrenal gland, blood calcium and phosphorous determinations in relation to parathormone, etc.

### HORMONAL TESTS FOR PREGNANCY

**Principles.**—1. The placental (chorionic) tissues apparently produce or contain two hormones, namely (a) estrone or theelin and (b) estriol or theelol. Collip, employing acetone as an extracting agent, believes that these constitute a single hormone differing in its physiological properties from the two gonadotropic hormones of the anterior lobe of the pituitary gland (prolan A and prolan B) and therefore designated as the anterior-pituitary-like hormone (A.P.L.) or the pregnancy urine factor (P.U.). This opinion has been widely adopted. It is also produced in hydatidiform mole, chorionepithelioma and malignant tumors of the testes with special reference to teratoma.

2. Tests for the anterior-pituitary-like hormone may be conducted with urine employing immature female white mice or rats according to the Aschheim-Zondek method<sup>1</sup>; likewise, with urine or serum employing immature female rabbits, according to the method of Friedman.<sup>2</sup>

3. *Positive reactions* by either method are indicated by the production of large hemorrhagic follicles or the presence of corpora lutea either enclosing the ovum or resulting from ruptured follicles. They occur in pregnancy as early as 5 to 14 days after conception and especially toward the end of the first month, with an accuracy of about 98 to 98.5 per cent.

4. Possible errors in the interpretation of tests may result in *falsely positive* reactions due to early menopause, hyperthyroidism, ovarian cysts, endometrial hyperplasia, uterine carcinoma or primary ovarian failure when pituitary compensation has occurred. The reactions under these circumstances, however, are generally limited to the first phase, characterized by ripening of the follicles and estrus in mice without hemorrhages into unruptured follicles or luteinization of the follicles.

5. In a *negative reaction*, by either the Aschheim-Zondek or Friedman tests, the ovaries are pure white or light pink although large mature follicles may be observed. In young rabbits, not old enough to produce a positive reaction, the ovaries may be narrow and flat and have an opaque appearance which may give rise to falsely negative reactions. The uterine horns are pure white. Otherwise, negative reactions indicate (a) the absence of pregnancy and other conditions giving positive reactions; (b) in a known pregnancy the death of the fetus except when living placental tissue is still present; (c) missed abortion and ectopic pregnancy following death of the placental tissue, or (d) the performance of the test too soon after conception. Negative reactions occurring in tests with urine voided less than 10 days after the first missed period are not dependable and indicate a repetition of the test later on unless a positive reaction is observed.

6. The hormone slowly decreases and disappears from the urine during the first week after parturition. When the reaction is negative after having been positive, fetal death is indicated although positive reactions may occur for 2 to 6 weeks if functionally active chorionic tissue is present, as (a) in about 50 per cent of ectopic pregnancies followed by negative reactions in about 3 weeks after the onset of vaginal bleeding; (b) possibly in missed and incomplete abortions as long as living placental tissue is present; (c) in hydatidiform mole; (d) in chorionepithelioma which should always be suspected when positive reactions occur longer than 2 weeks after the delivery of a hydatidiform mole, and (e) in men with teratoma, embryonal carcinoma and chorioncarcinoma of the testicles. When negative reactions following the surgical removal of hydatidiform mole, chorionepithelioma or malignant testicular tumors are followed in some weeks or months by positive reactions, recurrences or metastases are usually present.

**Aschheim-Zondek Test.**—1. A specimen of urine, not necessarily catheterized, is collected in the morning before breakfast. The fluid intake of the patient should be restricted during the evening preceding the collection of the specimen of urine in order to obtain concentration of the hormone. The bottle or other vessel in which the urine is collected should be cleaned with soap and water and dried thoroughly (alcohol should not be used). If the specimen is to be mailed to the laboratory, a drop of tricresol (lysol) per ounce of urine acts as suitable preservative which does not destroy the hormone. Properly preserved specimens do not lose their hormonal potency for at least 6 days.

2. If the urine is alkaline in reaction it is rendered slightly acid with a few drops of 50 per cent acetic acid.

3. A urine that is clear need not be filtered; one that is cloudy due to phosphates, urates, pus, or blood should be filtered. A cloudy urine, due to bacteria, should be detoxified as follows: 30 cc. of urine is added to 90 cc. of ether in a separatory funnel and shaken vigorously for 3 to 5 minutes. (Ether removes simultaneously practically all of the toxic substances in addition to the estrin present in the urine of pregnant

women). The urine is separated, filtered and allowed to stand in the open air in a large casserole for 1 hour. Add 0.9 gm. of glucose to the urine and dissolve.

4. The urine should be kept in a refrigerator between injections and gently warmed to 37° C. in a water bath before injecting. The temperature of the water bath should never exceed 50° C. since a temperature of 60° C. will destroy the hormone.

5. Use 5 immature female white mice weighing from 5 to 7 grams. Inject the urine subcutaneously, giving 0.2 cc. to No. 1, 0.25 cc. to No. 2, 0.3 cc. to Nos. 3 and 4 and 0.4 to No. 5.

6. About 90 to 100 hours later kill the mice with illuminating gas and inspect the ovaries with a hand lens. Normal immature ovaries are pinhead in size and pale in appearance. If the ovaries of all of the animals are found to be normal, the reaction is *negative*. A *positive* reaction is indicated by enlargement of the ovaries to two or three times normal size, and by minute yellowish protrusions of corpora lutea, or cyanotic protrusions which are due to hemorrhages into a follicle or a corpus luteum. There is also often swelling and hyperemia of the uterus.

**Kelso Modification of the Aschheim-Zondek Test.**<sup>3</sup>—1. Female rats, not younger than 22 nor older than 40 days, are used for test. These rats weigh between 30 and 65 grams. The females do not go into the normal period of puberty until they are 7 to 8 weeks old, so 40 days is a safe upper limit for premature development of the ovary due to the hormone in the urine. In rats over 40 days the animals are liable to show spontaneous ovarian maturity and give false positive results.

2. Inject 2 rats subcutaneously with 1 cc. of urine at 9 A.M., 1 P.M. and 5 P.M. If the urine is pale in appearance and the specific gravity is below 1.010, inject 2 cc. in each dose.

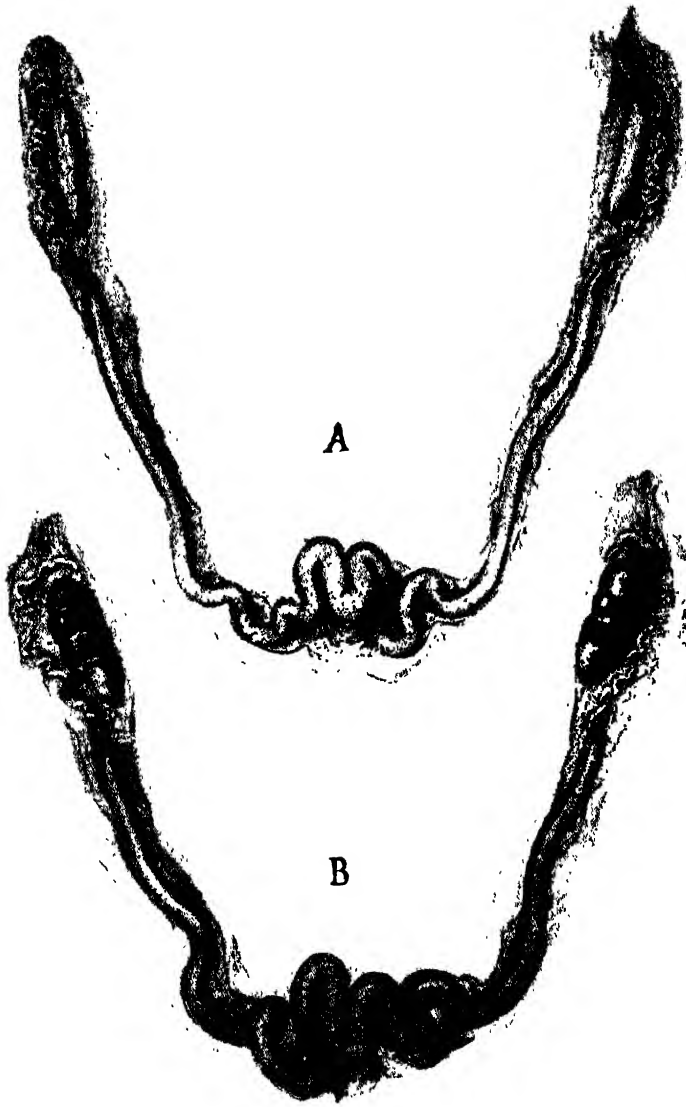
3. About 24 or 30 hours after the first injection kill the animals. The animals are then tacked down on board and an abdominal incision is made to expose the entire cavity. The intestines are lifted back toward the head of the animal which allows a clear view of uterus, tubes and ovaries. The uterus and tubes are ignored, but especial attention is centered on ovaries; a *positive* reaction is present when the ovaries are enlarged and hyperemic. Both must be present before a positive diagnosis can be made. Either one alone is insufficient for a positive diagnosis. Hemorrhagic follicles may be present, but these only indicate a strongly positive reaction and are never present unless the ovaries are enlarged and hyperemic. A *negative* reaction is indicated by small, pale ovaries.

**Friedman Test.**—1. Collect urine as for the Aschheim-Zondek test and filter.

2. Inject 2 doses of 10 cc. each at about 6-hour intervals in the ear vein of a female rabbit which should be not less than 17 weeks old and should weigh not less than 1500 grams. One dose of 13 to 15 cc. is probably preferred. Sterile precautions are not necessary, but the urine should be warmed to room temperature before the injection. The rabbits should be obtained from a reliable breeder. They should be separated at weaning and kept in individual cages for 3 to 4 weeks before use. Reinhart<sup>4</sup> prefers mature, nonpregnant rabbits not less than 6 months old and weighing not less than 2.5 kilograms. They should have had a litter and been isolated for 3 to 4 weeks preceding the test. Such mature rabbits should receive 2 injections of 15 cc. of urine.

3. Anesthetize the rabbit 48 hours after first injection and examine ovaries. Ether or intravenous injection of sodium amytal (60 mg. per kg. of body weight in distilled

## PLATE VIII



### A HORMONE TEST FOR THE DIAGNOSIS OF EARLY PREGNANCY

*A*, Bicornate uterus, tubes, and ovaries of a fourteen-week rabbit, thirty hours after injection of 7 cc. of urine from a non-pregnant patient. This demonstrates a negative result, with no changes occurring in the ovaries.

*B*, Bicornate uterus, tubes, and ovaries of a fourteen-week rabbit, thirty hours after injection of 7 cc. of urine from a pregnant patient. This demonstrates a positive result from an approximate five weeks' pregnancy, showing the presence of numerous corpora lutea and corpora hemorrhagica.

(From P. F. Schneider. *Surg., Gynec. & Obst.*, Jan. 1931.)



water) 30 to 40 minutes before operation are recommended as anesthetics or local, using 2 per cent novocain. Place rabbit on operating board and elevate the caudal end of the animal to permit a better view of the genital organs.

4. If negative, the ovaries remain small in size and show no change (see A in Plate VIII); if positive, from 1 to 14 corpora hemorrhagica and corpora lutea are found in each ovary. In some instances a positive result has been obtained in 12 hours and Schneider<sup>6</sup> recommends injecting 2 rabbits in cases in which a diagnosis of pregnancy might influence a decision regarding operation. The first rabbit is then examined at 12 to 24 hours and the result checked by examination on the second rabbit at 48 hours without loss of time. If one ovary appears negative, the other must be examined, but if one ovary appears positive, the other need not be inspected. It is well to remember that some rabbits, though fortunately only few, are refractory to the injection of gonad stimulating factors and fail to show the characteristic changes in the ovaries even when injected with the urine of known pregnant patients. This seems to be more often the case in young rather than in older rabbits. Such rabbits have commonly small and poorly developed ovaries. Therefore, if the ovaries appear small and the follicles indistinct, it is advisable to inject the rabbit with 10 cc. of urine from a known pregnant patient, and to withhold the final report until the rabbit was shown to react to a known positive urine. If it remains negative, then a new rabbit has to be injected.

Negative rabbits can be used again immediately. Rabbits with positive results can be used after an isolation of 10 to 14 days. The abdominal wound should be closed in two layers.

5. Rabbits may die during the test, due to too rapid injection of the urine, the presence of aspirin or quinine in the urine, the use of inbred rabbits or the injection of highly acid or alkaline urine. (The reaction should be adjusted by the addition of decinormal hydrochloric acid or decinormal sodium hydroxide to give a pH between 6.8 and 7.4.)

6. Under the circumstances the test may be conducted with *serum*, as described by Hoffmann as follows: (a) Collect 25 cc. of blood at any time during the day; (b) centrifuge and separate the serum; (c) add an equal amount of ether, shake and separate the serum; (d) inject 10 to 13 cc. of serum into the marginal vein of a virgin female rabbit not less than 17 weeks old and weighing not less than 1500 grams; (e) autopsy the animal and examine the ovaries about 24 to 30 hours after injection.

**Frog Test.**—This test, described by Weisman, Snyder and Coates (*West. Jour. Surg. Obstet. and Gyn.*, 50: 557, 1942), is based upon the observation that when mature healthy, female, African clawed frogs (*Xenopus laevis*, Daudin) are injected with the concentrated urine of pregnant women, the chorionic gonadotropic hormone produces an extrusion of eggs within a period of 4 to 12 hours. The mature female *Xenopus* carries eggs throughout the year, only extruding them at mating or after the injection of hormones peculiar to pregnancy. The spontaneous extrusion of eggs is stated not to occur. The technic of the test, which is stated to have an accuracy of 99.6 per cent in pregnancy, is as follows:

1. Only mature, female, healthy and well nourished animals imported from South Africa, can be used.\* Standardized animals—those which have reacted previously by

\* The animals may be obtained from Paramount Aquarium, Inc., 61 Whitehall Street, New York, New York.



egg extrusion after the injection of pregnant urine—should be used to attain the maximum of accuracy. Stock frogs are kept in tanks carrying water at a depth of about 3 inches from the bottom maintained at room temperature ( $70^{\circ}\text{F}$ ,  $\pm 5^{\circ}$ ). The animals are fed small strips of beef heart, calves liver, and garden worms (if available) twice each week. Tank water should be replaced by fresh water of the same temperature prior to each feeding and 24 hours after each feeding. *The animals should not be fed for at least 24 hours prior to testing* since the injection of urine causes regurgitation of partially digested food which may interfere with the visualization of the eggs and the correct reading of the test.



FIG. 132A—SITE OF INJECTION IN THE FROG TAIL FOR PREGNANCY

Improved method of injection. Small thin needle inserted at right angle to mid-dorsal line (Courtesy of Drs. Weisman, Snyder and Coates)

2. Four ounces of morning urine are required of which 80 cc. are used. To the 80 cc. add 160 cc. of acetone. Mix thoroughly and allow to stand for about 15 minutes for the precipitation of proteins and hormones. Decant the supernatant fluid and save for redistillation. Allow the precipitate to dry, which may be hastened by fanning. Add 2 cc. of distilled water to the dry precipitate, stir thoroughly and centrifuge. Remove the supernatant fluid and adjust to pH 5.5 (nitrazine paper, Squibb) with 10 per cent sulphosalicylic acid.

3. With a small syringe fitted with a short insulin-type needle inject 1 cc. into the

craniolumbar lymph space on the back of the animal (Fig. 132A). This lymph space is reached by thrusting the needle superficially into the left thigh muscle, directing the point medially to the midline approximately  $\frac{1}{2}$  inch above the upper cloacal fold. Throughout its course, the needle should be seen clearly just beneath the skin. A firm gentle thrust carries it through connective tissue into the lymph space. Great care must be exercised against puncturing a lung which usually results in the death of the animal (Fig. 132B).

4. After injection, place the frog in a small tank or jar carrying about 3 inches of water and fitted with a platform of half-inch wire mesh about 1 inch above the bottom to prevent the animal from devouring its own extruded eggs. The container should be kept covered with a glass plate allowing access of air, but fastened or weighed down to prevent the escape of the frog.

5. Observe the animal at intervals after the first 4 hours. The extrusion of eggs constitutes a positive reaction (Fig. 132C). This may occur as early as 4 hours after injection, but ordinarily occurs between 6 and 12 hours. If, after 18 hours, no eggs are visible in the tank, the test may be considered as giving a negative reaction. If two animals are used for the test (advisable), one may extrude earlier than the other.

6. Animals giving negative reactions may be used again after a rest period of 1 week. Animals giving positive reactions may be used again after a rest period of 4 weeks. After each positive reaction it is advisable to burn off eggs adhering to the wire mesh platform with a Bunsen burner.

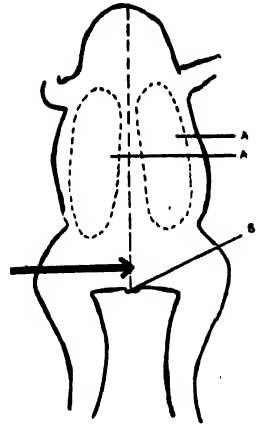


FIG. 132B.—DIAGRAMMATIC ILLUSTRATION OF LUNG ANATOMY AND SITE OF INJECTION

A, the lungs; B, the cloaca; C, the site where urine concentrate is injected. (Courtesy of Drs. Weisman, Snyder and Coates.)

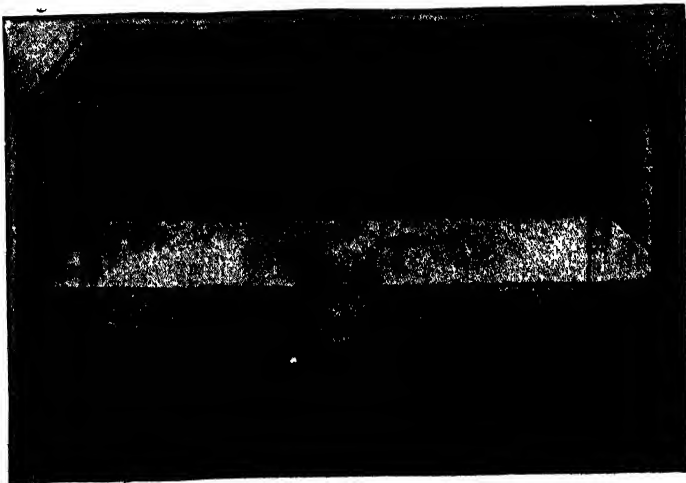


FIG. 132C.—POSITIVE XENOPUS REACTION WITH PREGNANCY URINE  
(Courtesy of Drs. Weisman, Snyder and Coates.)

### QUANTITATIVE ASCHHEIM-ZONDEK TEST FOR HYDATIDIFORM MOLE AND CHORIONEPITHELIOMA

Inject groups of 5 mice with 6 doses of 0.5 cc. each of urine diluted 1:10, 1:50, 1:100, and 1:1000. The technic<sup>6</sup> is the same as for the qualitative Aschheim-Zondek test. If the dilution of 1:10 gives a positive result then no less than 3,330 mouse units of hormone are assumed to be present per liter of urine, with corresponding higher values if the higher dilutions give positive results. A negative test does not exclude chorionepithelioma.

### QUANTITATIVE ASCHHEIM-ZONDEK TEST FOR TERATOMA OF TESTES

In about  $\frac{1}{3}$  of these tumors the amount of hormone eliminated in the urine is less than 2000 mouse units per liter. To recognize these cases the urine must be concentrated according to the method of Ferguson.<sup>7</sup>

**Preparation of Concentrate.**—1. The fresh morning specimen of urine is filtered if cloudy, and if alkaline is rendered faintly acid to litmus paper with a few drops of weak acetic acid.

2. Add 100 cc. of 95 per cent alcohol to 20 cc. of this urine in a graduate and mix by inverting several times. The mixture is allowed to stand overnight, the hormone separating out in the precipitate.

3. The following morning the supernatant fluid is siphoned off, leaving about 20 cc. of fluid over the precipitate. This is centrifuged for 5 minutes at 2000 r.p.m. The supernatant fluid is poured off.

4. Add 30 cc. of ether to the precipitate and mix by stirring with a glass rod for 10 minutes. Again centrifuge for 5 minutes and pour off the ether.

5. Distribute the precipitate around the bottom of the tube, using a glass rod, and allow it to dry. Add 4 cc. of distilled water; mix, and allow to stand overnight.

6. Centrifuge this mixture the following morning. The water now contains the hormone; it is pipetted off and kept in the refrigerator until used. This extract is a 5x concentrate of the fresh urine. Stronger extracts may be made by modifying the method.

Six mice are used. Three are given 5 doses of 0.1, 0.2, and 0.4 cc. of fresh urine and the other three similar doses of the concentrate.

According to Ferguson, there is a direct relation between the embryonal character of the tumor and the quantity of the excreted hormone. The highest amounts are found in chorionepithelioma, less in the embryonal adenocarcinoma and least in the teratoma of adult type. It disappears after successful removal of the tumor, decreases in quantity after irradiation, increases in recurrences and in the presence of metastases. However, further studies are needed to confirm those views and it must be remembered that a negative hormone test does not exclude malignancy of the testes.

### TESTS FOR ESTRIN

**Principles.**—1. Estrin, or estrone, is also known as the estrogenic hormone. It is produced by the graafian follicles and corpora lutea providing the ovaries have been stimulated by prolactin A, one of the two gonadotropic hormones produced by the

anterior lobe of the pituitary gland. Estrin not only supervises the first or proliferative half of the menstrual cycle, but governs the growth and development of the genital tract and the secondary sex characteristics. During pregnancy its secretion is greatly increased for the purpose of preparing the uterus for the effects of pitocin in parturition as well as promoting the development of the mammary ducts, the process being completed by the action of progesterin in developing the acini and by prolactin (the pituitary lactogenic hormone) which actually stimulates the secretion of milk. Estrin is divisible into three variants, namely, (a) estradiol, (b) estrone (theelin) and (c) estriol (theelol).

2. In the urine its variants are present in the form of free and combined estrones. They occur in the urine of children in amounts up to 50 international units per day, with larger amounts at puberty. Only very small amounts are present in the urine of adult males but women during menstrual life may excrete about 1500 units per month. However, it may be found in the urine of females some time after menstruation has stopped but is very low during old age. Its failure to appear in the urine of the mature female is indicative of ovarian failure.

3. An *increase* of estrin normally occurs between the time of ovulation and the onset of menstruation. It is also greatly increased during pregnancy, beginning about the eighth week. It is also frequently increased in follicular cysts of the ovary, adrenal cortical adenoma, Cushing's syndrome and granulosa cell tumors. An increase is also sometimes found in sterility, suggesting the possibility of habitual abortion as one of its causes. It causes inhibition of ovulation, endometrial hyperplasia and menorrhagia. A *decrease* may result in delayed puberty, sexual infantilism, amenorrhea or hypomenorrhea. A decrease may be observed in about 70 per cent of cases of obesity, suggesting that primary or secondary hypo-ovarism may be important in its etiology and especially after the menopause.

**Urine Test.**—1. Collect 24-hour urine and acidify with 15 volumes per cent of hydrochloric acid.

2. Boil the mixture (dark purple color) under a hood for 10 minutes to transform the bound estrin by hydrolysis into the free forms.

3. Allow to cool, add  $\frac{1}{3}$  volume of benzene and shake for 2 hours.

4. Separate the benzene and break up the emulsion by filtration.

5. Place the benzene extract in a distilling flask over an electric heater and reduce to about 10 cc.

6. Evaporate to dryness in an evaporating dish under a fan.

7. Mix the oily residue with 15 cc. of olive oil.

8. Give immature white female mice (18 to 21 days old and weighing 8 to 12 grams) a subcutaneous injection of 0.5 cc. once a day for 3 days in succession.

9. Examine the mice 96 hours after the first injection. If the vaginal opening is patent, prepare smears of the vaginal secretions and examine the cells. A *positive reaction* (estrus) is indicated by the presence of only flat, large, irregularly shaped, cornified epithelial cells with very small nuclei. Many occur in clumps with irregular patterns. There is little or no mucus, few or no leukocytes and no oval cells. A *negative reaction* is characterized by closure of the vaginal opening and the presence of mostly leukocytes with small amounts of mucus in smears. If the vaginal opening is patent but the smear dubious, the animal may be killed and the weight of the uterus determined. A weight of over 15 mg. is considered a positive reaction.

**Mack Vaginal Smear Test.**—As shown by Mack and his colleagues,<sup>8,9</sup> the vaginal epithelial cells of women with actively functioning ovaries are preponderantly squamous in character and have a high glycogen content. In hypo-ovarism or menopause the cells contain little or no glycogen. When estrogenic therapy is effective in hypo-ovarism or menopausal patients, the glycogen content of their vaginal cells is markedly increased. The glycogen content is determined by staining with iodine as follows:

1. Prepare vaginal smears on slides and allow to dry.
2. Place the slides face down over a shallow dish containing Lugol's solution for 2 or 3 minutes.
3. Examine macroscopically. Smears containing a normal amount of epithelial glycogen are of a deep brown color. Smears containing a deficiency of epithelial glycogen are of a lemon-yellow or very light brown color; microscopic examinations show yellow, glycogen-poor squamous cells, leukocytes and amorphous debris. The cells are smaller in size than normal and more rounded in shape than in women with actively functioning ovaries. This method is sufficiently sensitive to differentiate between glycogen values indicative of estrogen sufficiency and various stages of glycopenia corresponding to clinical estrogen deficiency. It is particularly useful in relation to estrogenic therapy. A method employing smears of vaginal secretions stained with a combination of Ehrlich's hematoxylin, eosin, and water soluble blue has been described by Papanicolaou (*Am. J. Anat.*, 52: 519, 1933).

### TESTS FOR THE GONADOTROPIC HORMONES

**Principles.**—1. The gonadotropic hormones secreted by the anterior lobe of the pituitary gland are two in number. One causes ripening of the follicles of the ovary with the production of estrin in the female and the proliferation of the epithelium of the seminiferous tubules of the testes in the male; it is called prolan A. The second stimulates the production of progesterin by the corpora lutea and their luteinization in the female and the stimulation of the interstitial tissue of the testes of the male; it is called prolan B.

2. These hormones appear in the blood and urine at about the time of puberty. They then disappear from the urine of the male but occur in the urine of the female just before ovulation. The amount present in the blood of normal and nonpregnant females is too small for determination. It may be increased, however, in primary ovarian weakness or true hyperpituitarism with ovarian hyperfunction. In pregnancy the blood carries large amounts of both hormones and especially prolan B. The quantitative determination, therefore, of the gonadotropic hormones may be of value in differentiating between primary and secondary gonadal disorders as in amenorrhea and impotency.

3. Both hormones are likely to be increased along with diminished estrin in both the blood and urine of castrated women as well as during and after the menopause due to hypogonadism. An abnormal increase in nonpregnant women is evidence of hyperpituitary function as sometimes occurs in hyperthyroidism and hyperadrenalism. A marked decrease or persistent absence of them, on the other hand, is indicative of hypopituitary function as seen in juveniles or the consequence of severe infections. About 90 per cent of cases of amenorrhea and oligomenorrhea have shown abnormal

hormone titers with about 50 per cent falling definitely into the class of hypogonadism with increased prolan excretion. Hypopituitarism is also sometimes responsible for sterility while obesity in women may show an increased excretion of the gonadotropines indicative of a primary hypo-ovarium.

4. Examinations of male urine for the gonadotropic hormones is conducted only occasionally. Only increased values are regarded as significant as for example, after castration, severe chronic orchitis and functional impotency.

**Urine Test.**—This test, which has been recommended by Levin and Tyndale<sup>10</sup> and Katzman,<sup>11</sup> also involves the use of immature female mice in determining their response to the gonadotropic hormones by weighing the uterus and examining for vaginal estrus, as in the urine test for estrin described above.

1. Collect a 24-hour specimen of urine.
2. Adjust the reaction to pH 5 with dilute hydrochloric acid (mustard green to nitrazine paper).
3. To each 100 cc. add 2 cc. of a 20 per cent solution of tannic acid. Add the tannic acid slowly, stirring constantly.
4. Allow the precipitate to settle for  $\frac{1}{2}$  to 2 hours in the refrigerator.
5. Decant the supernatant urine and discard. Wash the precipitate 3 times with 80 per cent alcohol, once with 95 per cent alcohol, and twice with acetone.
6. Dry the precipitate under an electric fan.
7. Suspend the precipitate in 10 cc. of distilled water and adjust to pH 9-10 (pink to phenolphthalein) with normal sodium hydroxide solution.
8. Inject immature female mice (18 to 21 days old and weighing between 8 and 12 gms.) subcutaneously with 0.5 cc. once a day for 3 days in succession.
9. Examine the mice 96 hours after the first injection. If the vaginal opening is patent, examine smears for estrus as described above in the urine test for estrin. If the vaginal opening is closed, the animal is regarded as negative. If the vaginal opening is patent but the smear is dubious, kill the animal and weigh the uterus. A weight of over 15 mg. is considered a positive reaction.

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# BACTERIOLOGICAL, MYCOLOGICAL AND VIROLOGICAL METHODS

## METHODS FOR THE COLLECTION AND HANDLING OF MATERIAL FOR BACTERIOLOGICAL EXAMINATIONS

**Principles.**—*Not infrequently bacteriological examinations are rendered entirely valueless by faulty methods in the collection and handling of material.* The subject, therefore, is one of considerable importance in which practicing physicians especially require the advice and guidance of bacteriologists. The chief points may be summarized as follows:

1. Obtain the material as free as possible from contamination.
2. As far as is possible, *obtain exactly what is desired to be examined.* For example, in culturing pus from the ethmoid or sphenoid sinuses, a mere swabbing of the nose is not satisfactory if pus can be obtained direct from the infected areas by a rhinologist.
3. Make *smears that are neither too thin nor too thick.* If only small amounts of material are available, one or two smears about the size of a dime are much better than larger ones spread out so thinly that it is difficult to decide on which side of the slide they have been made. The common practice of covering a large amount of material on one slide with a second is mentioned only to be condemned as filthy, potentially dangerous for laboratory workers, and usually entirely unsatisfactory for examination.
4. *Specify whenever possible the kind of examination to be made.* This is especially important in the case of feces and sputum. For example, if the former are submitted solely for examination for typhoid or dysentery bacilli, the request should be so stated in order that the laboratory may employ the special methods required. At least, the laboratory should be informed of the suspected infection in order to avoid unnecessary, expensive and time-consuming examinations.
5. *Choose the proper culture medium* if cultures are to be made. For example, if streptococcus or pneumococcus infection is suspected in a chronic otitis media, cultures of pus on plain agar may fail and show only the more rapidly growing organisms, such as staphylococci, diphtheroid bacilli, etc.
6. If mixed infection is suspected (as is usually true in chronic infections) prepare cultures on plates (blood agar recommended) instead of on slants; or submit the material itself or a swab for inoculation of plates in the laboratory.
7. Avoid soiling and contamination of containers, especially in the collection of sputum and feces.
8. Deliver material as quickly as possible to the laboratory after collection. This is

particularly important in relation to swabs because drying may result in the destruction of delicate microorganisms. In the case of unavoidable delay, material should be kept in a refrigerator since microorganisms will survive considerable period of time under such conditions, while cultures may be left overnight at room temperature without harm before incubation.

9. If contamination has occurred or if a defective method of collection has been used influencing the accuracy of the examination, as, for example, submitting smears that are too thin or cultures made on a wrong medium, to report the facts in order to guard against erroneous results and conclusions. For example, a culture of a sore throat on plain agar may show only staphylococci but does not exclude the possibility of diphtheria; or a culture of the eye on plain agar may show nothing but a staphylococcus and fail to grow streptococci or pneumococci if present.

10. With the exception of diphtheria (where the use of Löffler's blood medium is recommended) the most useful routine medium is blood agar plates. This medium will grow the less hardy organisms, including the hemoglobinophilic group, and thin spreads on plates lessen the chances of slowly growing organisms being overgrown by the hardier, rapidly growing ones.

11. As a general rule sterile swabs are better than platinum wires for securing material, as larger amounts of material are obtained.

12. Material delivered to the laboratory should be kept at a low temperature if impossible to examine promptly. This minimizes drying, reduces the multiplication of contaminating bacteria and even *B. influenzae* and other delicate organisms can withstand low temperatures for long periods of time. Likewise material received in the laboratory should be placed in a refrigerator until plated or otherwise examined.

## COLLECTION OF PUS FROM ABSCESES AND ULCERS

1. It is preferable to obtain pus at the time an abscess is incised or soon after spontaneous rupture.

2. The surrounding skin should be cleansed with an antiseptic like alcohol, bichloride or metaphen solution.

3. The pus should be gently expressed and collected on sterile swabs.

4. The infection is usually staphylococcic and cultures may be made on ordinary agar or broth, although blood agar is preferred.

5. Smears are helpful but not required for diagnosis.

6. In suspected *anthrax* of the skin (malignant pustule), smears should be made of the lesion and particularly of the serous contents of vesicles; cultures should be prepared on slants of plain or blood agar.

7. In suspected *tularemia* (ulcers on fingers), smears are useless. Cultures may be made on coagulated egg yolk or blood-glucose-cystin-agar. It is better to inoculate guinea-pigs with material.

8. In suspected *granuloma inguinale*, smears alone are required.



**COLLECTION OF MATERIAL FROM THE EYES**

1. It is advisable to prepare smears and cultures at a suitable stage of the disease. As a general rule, this is during the period in which the disease is developing, or is at its height. The actual causal agent can disappear rapidly, but the discharge lessens more slowly. In the stage of regression, the primary agent may not be found, but only staphylococci, diphtheroid bacilli, etc.

2. In conjunctivitis an effort should be made to avoid the collection of secretions in contact with the angles or margins of the lids unless angular conjunctivitis or blepharoconjunctivitis are present. In dacrocystitis, an effort should be made to secure fresh pus by expression.

3. In making smears and cultures it is generally advisable to first remove excessive exudates with saline solution or sterile gauze in order to secure microorganisms located in the epithelium. For this purpose it is necessary to first anesthetize the eye by the local instillation of sterile 4 per cent solution of cocaine or a 1 per cent solution of pontocaine followed by gentle scraping of the conjunctiva or cornea with a sterilized platinum spatula or von Graefe knife. Otherwise a small, sterile cotton swab may be used after it has been dipped in broth medium. Swabs, however, are not suitable for the preparation of smears to be examined for the intracellular inclusion bodies of the viral infections.

4. In corneal infections great care is required for avoiding injury of the tissues with the spread of infection. The cornea should be anesthetized and kept perfectly quiet. Superficial swabbings may be unsatisfactory. The point of a sterile von Graefe knife, platinum spatula or needle is generally preferred for obtaining material as the causal microorganism is likely to be deeply located.

5. Material from the anterior chamber may be aspirated with a small sterile syringe and needle.

6. Portions of the iris removed by iridectomy should be placed at once in glucose hormone broth suitable for the cultivation of streptococci and pneumococci (pH 7.4 to 7.6).

7. *Properly prepared smears are always of great value in all eye examinations* as they may show the presence of microorganisms failing to grow in culture media. At least 2 should be made. Avoid making smears too thin or too thick; smears the size of a dime are large enough.

8. In cultures of styes, plain agar may be used because they are caused by staphylococci. Otherwise, however, enriched media, like blood agar, are always required. In suspected tularemia, brucellosis, tuberculosis, etc., special media are required.

9. Smears and cultures should not be made within 4 hours after irrigation or instillation of disinfectant solutions. Indeed, it is better to wait for 12 to 24 hours if conditions permit.

10. Enucleated eyes should be seared or dipped momentarily in boiling water or a disinfectant solution for surface disinfection before being opened with a sterile knife or scissors for the purpose of preparing smears and cultures of the iris, lens, humors or uveal tract.

11. Darkfield examinations are of great value in the diagnosis of chancres, spirochillar infections, the detection of *Spirillum minus* in ocular infections of rat-bite

fever and for the detection of *L. icterohaemorrhagiae* and *L. canicola* in infectious jaundice or Weil's disease.

12. The inoculation of guinea-pigs with bits of tissue or secretions is indicated when oculo-glandular tularemia, brucellosis or tuberculosis is suspected; also the corneal inoculation of rabbits in suspected virus infections. Rabbits may be inoculated intratesticularly as an additional means for the detection of *T. pallidum*.

13. Smears for examinations of intracellular inclusion bodies in suspected virus infections should be stained by the Giemsa method.

14. Serological examinations for syphilis are indicated in suspected syphilitic keratitis, iritis, choroiditis, etc.; also complement fixation tests in suspected gonococcal iritis and iridocyclitis. Agglutination tests are indicated in suspected brucellosis and especially recurrent iritis, iridocyclitis or neuroretinitis sometimes due to *Br. melitensis*. Agglutination tests are also of diagnostic value in suspected infections with *L. icterohaemorrhagiae* or *L. canicola*.

### COLLECTION OF MATERIAL FROM THE NOSE, SINUSES, AND NASOPHARYNX

1. In culturing the nose, sterile swabs should be used and passed without touching the atrium. They may be first passed above and then below the lower turbinates to the nasopharynx if there are no obstructions.

2. Cultures should not be made within an hour of the application of antiseptics in order to avoid erroneous results.

3. Secretions may be blown into sterile gauze and portions picked up with sterile swabs (frequently unsatisfactory on account of contamination).

4. Material from infected sinuses should be collected by a rhinologist under direct illumination and with the aid of suction or other special methods for the purpose of securing a small amount of the material directly from the areas which are infected.

5. Cultures of the nasopharynx should be made through the mouth with curved wire swabs to avoid contamination with saliva. The West tube is useful but not necessary.

6. Plain agar should not be used except for cultures of pus from abscesses which are staphylococcal. Blood agar is recommended for routine use with Löffler's blood serum or hormone broth as second choice since rich media are required for the cultivation of streptococci, pneumococci, *Micrococcus catarrhalis*, meningococci, diphtheria bacilli and such organisms.

### COLLECTION OF MATERIAL FROM TONSILS AND FAUCES

1. When inflammatory exudates are present, as in diphtheria, follicular tonsillitis and Vincent's angina, collection with sterile swabs or a sterile platinum loop is sufficient.

2. The swabbing should not be too superficial but an effort made to secure material next to the tissues. This is especially important when diphtheria is suspected, as the bacilli are apt to be deeply located while the surface of a heavy exudate shows nothing but staphylococci. For this reason the first or primary culture may be negative for diphtheria bacilli unless a deep swabbing or a portion of membrane is secured.

3. Löffler's blood serum and blood agar are recommended for the preparation of cultures.

4. Smears on microslides are useful. *In Vincent's angina, smears only are required* as the organisms cannot be cultivated except by very special anaerobic methods.

5. In a bacteriological examination of the tonsils in relation to focal infection, it is advisable and recommended to obtain material from the crypts whenever possible as these are likely to be more satisfactory than surface swabbings. As a general rule these collections are best made by a laryngologist. Material may be expressed from the crypts or secured with the aid of a special sterile glass tube attached to a suction pump. A good method and one that may be conducted in the laboratory is first to make surface swabbings in different directions, as the flora may vary in different locations, followed by a culture of one or more crypts with a platinum loop bent at right angles.

6. Excised tonsils should be delivered in sterile gauze or saline solution immediately after removal. In the laboratory, they may be seared with a hot blade, dipped into boiling water or 70 per cent alcohol for surface disinfection, washed several times with sterile saline and laid open with a sterile knife or scissors. Cryptic material and bits of tissue are then secured and planted in brain-hormone broth or a similar enriched medium adapted for the cultivation of streptococci. Emulsions of tonsil and adenoid tissue may be prepared and cultured.

### COLLECTION OF SPUTUM AND BRONCHIAL SECRETIONS

1. Sputum should be collected in a sterile wide-mouthed bottle or vial with the minimum contamination of the mouth and saliva. As a general rule, morning sputum is to be preferred, the patient being instructed to brush the teeth with a boiled toothbrush and to wash the mouth with boiled water before coughing occurs.

2. In suspected *whooping cough*, sputum may be collected in this manner in the case of older children and adults. In young children, faucial secretions may be collected on swabs. Smears are of no value. Cultures should be made on glycerol-potato-blood agar adjusted by acetic acid to pH 5.0, and plates of this medium may be held before a child during a paroxysm of coughing for making "droplet" cultures.



FIG. 133.—TUCKER COLLECTOR

3. Sputum for examination for tubercle bacilli alone by smear methods need not be collected with these precautions. However, when cultures and guinea-pig inoculation tests for tubercle bacilli are to be conducted, they are helpful in reducing the degree of contamination.

4. Sputum to be examined for tubercle bacilli by *smear* alone may be collected in 5 per cent phenol although this is not necessary as the specimens may be autoclaved for sterilization before examination without damage to the morphology or tinctorial

properties of the bacilli. In hospitals the collection may be made in paper boxes which are later destroyed by burning, but these cannot be sterilized before examination and are not recommended. Patients should be instructed to carefully avoid contamination of the outside of containers, as the material may be dangerous to handle and bacteriologists are advised not to examine material delivered in such shape.

5. Specimens should be delivered as soon as possible and kept on ice or at a low temperature until examined. Specimens 24 hours or longer since collection are almost useless for bacteriological examination except for tubercle bacilli by the smear method; older specimens, and especially those kept at room temperature, deteriorate in value for guinea-pig inoculation and culture with increasing time.

6. Bronchial secretions aspirated with the Jackson bronchoscope are especially well adapted for bacteriological examination in cases of asthma, chronic bronchitis and bronchiectasis. The Tucker (Fig. 133) and Clerf (Fig. 134) collectors are very satisfactory. After use, the collector may be sent to the laboratory, or smears and cultures (blood agar or hormone broth preferred) prepared in the clinic.

### COLLECTION OF MATERIAL FROM THE TEETH AND GINGIVAE

1. For the bacteriological examination of *extracted teeth*, the following method is recommended: (a) Wall off the operative field with sterile cotton rolls. (b) Dry off the area about the tooth with sterile gauze and apply 3 per cent tincture of iodine with care to have it penetrate well into the gingival margin about the tooth. (c) Extract, and while holding tooth in the extraction forceps, sever the apex with a cutting forceps and drop it untouched into a sterile test tube or vial. (d) Deliver as soon as possible to the laboratory, where the fragment should be cultured in a rich broth medium like glucose-brain broth; or (e) the fragment may be dropped into a sterile screw top vial containing a small amount of sterile sand and gelatin-Locke solution. In the laboratory this should be shaken for 10 minutes to macerate the tissue as much as possible and the material transferred with a sterile pipet to tubes of glucose-brain broth and streaked on blood agar plates.

2. Cultures of the *socket* may be made by: (a) Walling off the tooth with particular care with sterile cotton rolls to prevent contamination with saliva or the tongue; (b) disinfecting the gums with tincture of iodine; (c) extracting with forceps with sterilized beaks; (d) curetting with sterile curet or with a sterile cotton swab removing material for inoculation in glucose hormone broth or on blood agar plates.

3. Numerous methods have been advised for *culturing the periapical region through the root canals with the tooth in situ* but the following is recommended as a simple one for routine use: (a) Isolate the tooth with rubber dam. (b) Sterilize the



FIG. 134.—CLERF COLLECTOR

coronal surface with a 3 per cent tincture of iodine. (c) Remove the filling with sterile instruments. (d) Remove filling or dressing in the root canal with sterile instruments. (e) Mechanically cleanse and dry the canal with sterile cotton and insert sterile paper points slightly moistened with sterile saline solution to absorb any moisture oozing into the canal; remove the points and drop them into a tube of glucose hormone broth. (f) If no moisture oozes in, pass a sterile, fine broach or pick through the canal and drop it into a tube of the medium.

A more elaborate method recommended by Rickert is as follows: The canals are first opened large enough to be readily accessible. The canal walls should be cleansed with alcohol or hydrogen peroxide. The treatment is introduced on an aseptic cotton point of a length not to exceed two-thirds the length of the canal; above this toward the occlusal orifice place a short section of the dry thickened end of a sterile cotton point; then above this place cotton moistened with sandarac varnish. The cavity is next sealed with either cement or temporary filling. In taking the culture, the tooth and adjacent teeth are isolated, dried and treated with tincture of iodine. The temporary stopping is removed and the cavity moistened with iodine; the sandarac varnish stopping is then removed and this portion cleansed with alcohol. The last dry pledget is then removed with a barbed broach and the dressing to be cultured is carefully withdrawn; it is seized just above the point of contact of the broach with sterile cotton pliers. The broach, cut off to the pliers and the remaining part of the point which is the apical end, is then introduced into the culture medium.

4. Smears are generally employed for the bacteriological examination of the *gingivae* or *gums*. Material should be especially collected from the sulci or pockets alongside of the teeth in infection of the periodontium. A stiff platinum loop or some other suitable instrument may be employed; or pus may be picked up with swabs after expression.

Smears may be prepared on microslides in the usual manner, allowed to dry and sent to the laboratory for staining and examination for spirochetes, endamebae, etc. When the secretions are scanty a drop of the patient's saliva or a drop of saline solution should be placed on the slide, the material added, and a smear prepared.

Wet preparations are very useful for examination for spirochetes and endamebae. In this case the patient must be sent to the laboratory or the examination made in the office of the dentist. The spirochetes are readily seen stained or unstained and darkfield examination is not necessary.

Cultures of the surface of the gums are hardly worth while because of inevitable contamination with saliva. But pus expressed from pockets is quite suitable for cultures if the surface is first disinfected with 3 per cent tincture of iodine. The pus is then collected on sterile swabs and sent at once to the laboratory or directly streaked over blood agar plates and then planted in tubes of enriched broth (brain-glucose broth is recommended).

### **COLLECTION OF MATERIAL FROM THE EAR AND MASTOID**

1. In culturing furuncles of the external auditory canal, the skin should be cleansed with alcohol and pus picked up with a small sterile swab. Plain or blood agar or Löffler's blood serum may be inoculated, as these infections are usually staphylococcic.

2. In otitis media the material is best collected by an otologist, as the external auditory canal should be cleansed, disinfected with alcohol and pus obtained on sterile swabs through a speculum and with illumination in order to guard against contamination.

3. In acute otitis media, the organism producing infection is generally obtained in pure culture; in chronic otitis media, two or more organisms are generally found.

4. The pus should be streaked on blood agar plates or inoculated in tubes of enriched broth like glucose-brain broth or glucose-hormone broth. It is a mistake to use plain agar, as this is not suitable for the cultivation of streptococci, pneumococci, etc. In acute otitis, cultures on Löffler's blood serum or slants of blood agar are sufficient, but in chronic otitis, blood agar plates should be used since the infection is generally mixed and rapidly growing organisms may readily overgrow streptococci and similar slowly growing ones. Smears on slides are also serviceable as their examination gives valuable information, especially in regard to the organisms one may expect to find in the cultures.

5. The same procedures are recommended in mastoid infections. Smears and cultures should be made at the time of operation on blood agar slants or plates or glucose-brain broth suitable for the cultivation of pneumococci and streptococci.

### COLLECTION OF CEREBROSPINAL FLUID, PLEURAL AND OTHER TRANSUDATES AND EXUDATES

*Cerebrospinal fluid* for bacteriological examination should be collected with particular care against contamination, especially if cultures are to be made. The presence of staphylococci in cultures is rather common but of no significance unless skin contamination can be definitely excluded. As meningococci, pneumococci and streptococci in spinal fluid tend to die out rapidly, especially meningococci, the *fluid collected in sterile test tubes should be sent as soon as possible to the laboratory* where large amounts (0.5 to 1.0 cc.) should be cultured on blood agar, sheep serum agar or similar enriched media. Smears are also of great value and may be prepared after the cultures have been made by smearing the fluid direct if it is purulent or after securing sediment by centrifuging.

Pleural, pericardial, joint and other fluids should be collected by aspiration with a sterile syringe fitted with a sufficiently large needle and under rigid aseptic conditions with particular reference to very careful preparation of the skin. Cultures and smears are then prepared as in the case of cerebrospinal fluid.

### COLLECTION OF BILE

1. The technic for collection of bile from the duodenum by nonsurgical drainage is extremely important as the bacteriological examination is almost without value unless the bile is collected with rigid precautions against the several sources of contamination. It is recommended that the method described on page 210, employing a special flask, be strictly followed.

2. A broth medium known to be suitable for the cultivation of streptococci should be employed; hormone broth with a pH of 7.4 to 7.6 is recommended.

3. About 20 drops of bile should be added to 150 cc. of medium.

4. If a plating method is to be used, bile may be collected in a special sterile vial or test tube.

5. Upon delivery of the specimen, 0.5 to 1.0 cc. of bile may be removed with a sterile pipet and plated on blood agar, the colonies being examined after 24 to 48 hours incubation. The broth method, however, is generally more satisfactory.

### COLLECTION OF STOMACH CONTENTS

Needless to state, special precautions are required in the collection of stomach contents for bacteriological examination, as follows:

1. The patient should be fasting for at least 18 hours and the teeth and gums carefully cleansed several times during this interval.

2. Beginning 1 hour before the test-meal, the mouth and throat should be gargled several times with a bactericidal solution like 1:1000 mercuraphen or mercuric chloride in 1:10 liquor antisepticus, followed by sterile water.

3. If the nose and postnasal space are infected, these parts should be cleansed to remove the secretions.

4. The test-meal should be sterilized.

5. For at least  $\frac{1}{2}$  hour, or better, 1 hour prior to the time set for removal of the stomach contents, the patient should be cautioned against the swallowing of saliva; a dental suction apparatus may be of aid.

6. The stomach tube should be sterilized and protected as much as possible against contamination during its passage. The tube should be pushed down rather than left entirely to the swallowing efforts of the patient in order to reduce the chances of swallowing saliva. Specimens for bacteriological examination are always best secured from "tube-broken" individuals.

7. The specimen should be collected in a sterile container and examined as soon as possible, being kept on ice if a delay is unavoidable.

### COLLECTION OF MATERIAL FROM FECES AND THE RECTUM

1. Feces should be passed directly into a quart-size Mason jar previously sterilized by boiling it, the rubber ring and the top for a few minutes before use. Or the patient may pass a stool into a basin previously sterilized by boiling, and a portion (especially feces with mucus) removed with a sterile spatula to a sterile wide-mouthed bottle or vial.

2. Cultures of the rectum for cholera and typhoid carriers may be made by cleansing the skin about the anus with soap, water and alcohol, followed by the introduction of a sterile cotton swab previously moistened with sterile broth or saline solution; or sterile vaselin may be applied to the anus and the finger, covered with a sterile rubber cot, inserted and swabs prepared from the cot. The swabs should be delivered promptly to the laboratory for inoculation of culture media.

3. In ulcerative colitis, cultures are best made with the aid of sigmoidoscope or proctoscope. The ulcers should be first cleansed and material obtained with sterile swabs which should be streaked over blood agar plates or a primary culture made in an enriched broth, like brain-hormone broth for the cultivation of streptococci, etc.

## COLLECTION OF BLOOD FOR CULTURES

**Principles.**—1. The detection of bacteria in the blood depends entirely upon blood cultures since they are too few for detection by the examination of stained smears. *Treponema pallidum* occurs in the blood during the primary and secondary stages of syphilis, but in too few numbers for detection by darkfield or other direct methods of examination. Furthermore, since they cannot be cultivated, detection depends entirely upon the inoculation of the testicles of rabbits, with blood but is not employed for diagnostic purposes. Some of the other pathogenic spirochetes, however, like those producing relapsing fever and rat bite fever, may be detected by direct microscopical examinations of the blood along with animal inoculation tests. Blood cultures and animal inoculation tests have also proven of value in the diagnosis of infectious jaundice due to *L. icterohaemorrhagiae*. None of the rickettsia or viruses, however, are ordinarily detectable by blood examinations.

2. A large number of methods have been proposed and the technic employed has considerable influence upon results. Aerobic methods are generally employed, but undoubtedly many falsely negative results occur because of failure to use *anaerobic methods*. It is true that in the deeper parts of broth cultures there is sufficient reduction of oxygen tension for the cultivation of micro-aerophilic organisms, but strictly anaerobic methods should be employed alone or in conjunction with aerobic methods in blood cultures on all suspected cases of puerperal sepsis, with special reference to hemolytic streptococci and *Cl. welchii*, as well as in all suspected cases of the post-operative septicemias in relation to hemolytic streptococci and organisms of the genus *Bacteroides*.

3. It is particularly important to add 5 mg. of *para-aminobenzoic acid* (P.A.B.) to each 100 cc. of medium in all blood cultures of individuals who are receiving sulfonamide therapy. Otherwise, streptococci and other microorganisms actually present may fail to proliferate with falsely negative results which can be not only misleading but actually disastrous in relation to sulfonamide therapy.

4. *Blood cultures are best made in fluid media* although it is true that the results are not quantitative in the sense of showing the number of microorganisms per cubic centimeter of blood. For this purpose plating 1 or 2 cc. of blood with agar-agar at the bedside may be employed; or 5 cc. of citrated blood may be delivered to the laboratory for plating purposes. But only 5 to 10 per cent of positive blood cultures employing a broth medium may prove positive by these plating methods. Consequently, the clinical value of the latter is sharply limited and never to be relied upon alone for diagnostic purposes; indeed, they are advisable only on special occasions in relation to sulfonamide or serum therapy.

5. Needless to state, the *kind of medium* employed is of great importance; the optimum pH is ordinarily from 7.4 to 7.6. Many media have been employed and especially hormone broth with 0.2 per cent glucose, but tryptose phosphate broth and the heart-brain broth of Kracke are particularly useful. Ordinarily flasks carrying 75 cc. inoculated with 5 to 6 cc. of blood are satisfactory. As a general rule it is advisable to collect blood at the height of fever whenever possible. It is helpful to know the kind of infection suspected clinically in order to choose the proper culture medium and method to be employed.

6. It would appear that more attention should be given to the matter of *choice of*



*vein* for securing blood for culture purposes. As a general rule a vein in the arm is employed, but apparently this is not always a good routine procedure. For example, it has been shown that in septicemia due to thrombophlebitis, blood taken from a vein directly draining the focus contains more organisms than venous blood removed from the arm because of less dispersion. It would seem advisable, therefore, to secure blood from a vein draining an infected area whenever feasible. Several investigators have reported that cultures of blood removed from the femoral artery have shown a higher incidence of positive results than duplicate cultures of venous blood. Whether or not the procedure is justified for ordinary diagnostic purposes, in view of its greater technical difficulties, cannot be stated, although it appears worthy of further trial. Methods for securing venous blood from adults and children are described and illustrated on pages 44 to 48.

7. *Rigid aseptic technic* is required not only to guard against contamination from the skin and air in the collection of blood, but likewise in making subcultures and all subsequent examinations.

8. The methods employed may be designated as: (a) *Qualitative* to determine whether or not bacteria are present; (b) *quantitative* to determine the approximate number of bacteria per cubic centimeter of blood; and (c) *massive* when large amounts of blood are cultured for the detection of small numbers of organisms, as in chronic infective types of arthritis.

9. When relatively large numbers of bacteria are present in the blood the cultures are usually positive within 24 to 72 hours of incubation; but when only small numbers are present, the cultures may require 10 to 21 days' incubation and no culture should be reported as "sterile" in less than 10 days.

10. The presence of *Staphylococcus albus*, *B. coli*, *B. proteus* and diphtheroid bacilli is frequently due to contamination and when found, the culture should be repeated before concluding that they were from the blood.

**Method for Collection of Blood.**—Prepare the skin as follows:

1. Wash thoroughly with hot water and soap.
2. Cleanse with alcohol and sterile gauze.
3. Apply a wet dressing of 1:1000 bichloride of mercury for at least 30 minutes.
4. Apply tourniquet and request the patient to vigorously open and close the hand in order to distend the veins.
5. Light an alcohol lamp.
6. Assemble a carefully sterilized 20 cc. Luer syringe with No. 20 gage needle.
7. Remove gauze dressing and apply tincture of iodine over a prominent vein.
8. Ask patient to keep hand clenched. Avoid touching the skin at the site of puncture. If necessary to palpate the vein wear sterile rubber gloves or cover finger with sterile gauze.
9. Make venous puncture and withdraw 10 to 15 cc. of blood. Ask patient to open hand. Release the tourniquet. Withdraw needle from the vein.
10. Inoculate the media after carefully flaming in alcohol lamp.
11. Remove iodine from the skin with alcohol. Apply flexible collodion and cotton or gauze dressing.

**Routine Method for Blood Culture.**—The following method can be recommended for routine cultures as it is suitable for most of the pathogenic bacteria producing bacteremia and septicemia and provides both a qualitative and quantitative culture:

1. Inoculate a flask of 150 cc. of glucose (0.2 per cent) hormone broth (pH 7.4 to 7.6) with 5 or preferably 10 cc. of blood. The Kracke heart-brain broth is also recommended. Other media may be used depending upon the infection suspected.

2. Place 5 cc. of blood in a test tube carrying sterile sodium citrate and rotate thoroughly to prevent coagulation. (These tubes are prepared by placing 2 cc. of a sterile 10 per cent solution of sodium citrate in distilled water in each. Place in incubator or water bath until evaporated to dryness. Replace cotton with boiled rubber stoppers. Each tube will contain 0.3 gm. sodium citrate sufficient for 5 cc. of blood.)

3. In the *laboratory* culture the citrated blood as follows: (a) Melt 2 tubes of plain or glucose agar in a water bath and cool to 42° C. (b) To one add 1 cc. of citrated blood and to the second 2 cc. with a sterile pipet. (c) Pour into 2 sterile Petri dishes and mix thoroughly; label each plate with amount of blood used. (d) Allow to harden and incubate covers down. (e) This method is better than inoculating tubes of agar and pouring plates at the bedside, as it usually permits more accurate measurement of the blood and especially if bubbles of air gain access to the syringe.

4. Incubate the flask and plates for 48 hours when a preliminary report should be made. With great care against contamination, prepare a smear of the supernatant broth and stain by the method of Gram. At the same time subculture about 0.5 cc. of the sedimented blood and broth on a slant of blood agar. Repeat every 2 or 3 days for 10 to 21 days if there is no growth before rendering a final report. If growths develop identify the organisms. If a growth appears in the plates, report the number of colonies per cubic centimeter of blood.

**Anaerobic Method for Blood Culture.**—If an anaerobic culture is desired, as is advisable in culturing the blood of suspected cases of puerperal septicemia, it may be prepared by planting 1 or 2 cc. of citrated blood at the bottom of a long narrow tube of Rosenow's brain-broth medium by means of a sterile pipet. Cover with sterile paraffin oil or vaselin. Such a culture gives partial oxygen tension or it may be incubated in a special jar in an atmosphere of hydrogen gas for more complete anaerobiosis. Incubate at least 5 days before preparing smears for examination. If no growth, continue incubation and examinations for at least 3 weeks.

**Method for Blood Clots.**—Specimens of clotted blood submitted in sterile Keidel or test tubes for serological examinations may be utilized for preparing blood cultures after the following method by Sellers and Morris:

1. After sufficient serum has been withdrawn aseptically for serological tests, transfer the remainder of the specimen, including the clot, into the barrel of sterile Luer syringe.

2. With the nozzle turned upward, expel the air with the plunger.

3. The nozzle is then held over the mouth of the flask of broth medium and the clot "spued" into the medium by pressure on the plunger. A thorough comminution of the clot is thus obtained releasing organisms trapped in the clot.

**Choice of Culture Medium.**—The glucose hormone broth recommended above is suitable for the cultivation of most pathogenic organisms producing septicemia as the staphylococci, streptococci, meningococci, gonococci, typhoid bacilli, etc. For other organisms special media may be required according to the infection suspected.

**Massive Methods for Blood Culture.**—If the routine method described above proves sterile when but few organisms are present, larger amounts of blood may be cultured by one of the following methods:

*Method of Cecil and Nicholls for Arthritis Cases.*—1. Twenty cc. of blood are taken aseptically from the arm vein of the patient, and placed in two sterile test tubes.

2. The blood is allowed to clot and placed in the ice box overnight.

3. In the morning the serum is removed and the clots transferred to bottles containing 50 cc. of beef-heart infusion broth with a pH 7.6. The bottles are then placed in the incubator at a temperature of 37° C., and left there for 1 month.

4. During the month subcultures are made at 5-day intervals on blood agar pour plates and in blood broth tubes. If at the end of 30 days the subcultures remain sterile, the sediment of the original bottle is examined for organisms by means of smears. Part is streaked on a blood agar plate, and part is transferred to fresh blood broth. If no organism can be demonstrated with this procedure, the blood is considered sterile.

5. All cultures and transfers are made under a hood in order to eliminate contaminations as far as possible. All contaminated cultures are discarded.

### COLLECTION OF URINE

1. Urine collected by voiding is generally unsatisfactory for bacteriological examinations except for the detection of tubercle bacilli. The possibility of errors due to contamination with *B. smegmatis* has been greatly overemphasized. Otherwise, however, urine is unavoidably and invariably contaminated with staphylococci, colon bacilli and other microorganisms. Consequently, reports on bacteria detected in urinary sediments in the course of ordinary routine microscopic examinations are of no clinical value.

2. Therefore, urine for bacteriological examinations should be collected by catheterization whenever possible. Great care is required in the technic not only for the purpose of avoiding contamination, but likewise for the prevention of accidental infection of the bladder. When this is not possible, and especially in the case of infants, the voided urine should be examined *immediately* after collection before contaminating microorganisms have greatly multiplied. Smears of sediment obtained by centrifuging and stained by the method of Gram will usually yield valuable information. Cultures in broth are of no value but plate cultures frequently show a preponderance of the infecting microorganisms.

3. Even when urine is collected by catheterization of the bladder, however, contamination may occur with microorganisms in the meatus and first portions of the urethra. Consequently, the clinical significance of such microorganisms as *Staphylococcus albus*, the colon bacilli and *B. proteus* is frequently difficult to determine. Contamination by these sources is much less likely to occur in the collection of urine from each kidney separately by ureteral catheterization, which is always advisable in the etiological diagnosis of infections of the kidneys whenever possible.

4. Catheters should be very carefully sterilized. Glass catheters are preferred for the removal of urine from the bladder of women. Patients should be instructed to retain urine for several hours at least so that the first ounce or two may be discarded before a specimen is collected in a sterile test tube or vial.

5. The meatus and neighboring parts should be carefully cleansed with tincture of green soap and water followed by the application of solutions of metaphen, merthiolate or some other suitable disinfectant.

6. In the case of children, small catheters must be used. In infants sterile test tubes may be fastened with adhesive tape over the penis, or over the urethral meatus in girls. Contamination, however, is unavoidable. For this reason the urine should be examined bacteriologically just as soon as possible after collection; 2 to 5 cc. are ordinarily sufficient.

### COLLECTION OF URETHRAL AND PROSTATIC SECRETIONS

1. Smears of urethral pus are ordinarily sufficient for the bacteriological diagnosis of gonorrhea. But the method of preparing them is of practical importance. At least two smears should be prepared with cotton swabs and rolled (not rubbed) upon slides, care being taken not to pass the swab over the same surface twice. If the amount is scanty, smears the size of dimes are sufficient, as very thin smears are unsatisfactory.

2. It is sometimes advisable to furnish the patient with slides and swabs along with instructions for preparing smears of morning secretions, collected upon arising and before urination.

3. Smears should be allowed to dry in the air. The practice of covering a heavy wet smear with another slide is very unsatisfactory.

4. When cultures are to be made it is advisable to cleanse the meatus and secure pus by urethral massage. This should be picked up with sterile swabs immediately streaked on a suitable medium like that of Pelouze, North, blood agar, etc., or the swabs may be washed out in a small amount of sterile ascites fluid in a test tube furnished with a sterile rubber stopper and at once forwarded to the laboratory for inoculation of media.

5. The examination of urine for gonococci in chronic urethritis is not very satisfactory, although staphylococci, colon and diphtheroid bacilli, etc., from the prostate gland are readily obtained. The prostate gland should be thoroughly massaged and the *first ounce or two of urine immediately passed collected* in one or two sterile centrifuge tubes and used for examination. Or the prostate may be massaged during urination. If cultures are to be made the urine should be secured by catheterization into sterile centrifuge tubes. Whatever method is used it is important to centrifuge the urine as soon as possible at high speed and the sediment should be streaked on a suitable medium and also examined by direct smears.

6. Bacteriological examination of prostatic secretions may be conducted by having the patient empty the bladder immediately before examination. The meatus is then thoroughly washed with soap and water. While the patient constricts the urethra just behind the glans penis, the prostate is thoroughly massaged until fluid collects behind the constriction. This is then collected in one or more sterile Petri dishes and plated on blood agar. Smears may be prepared for the Gram stain at the same time.

### COLLECTION OF VAGINAL SECRETIONS

1. Properly prepared smears are still of most value in the bacteriological diagnosis of gonococcus urethritis, vaginitis, Bartholinitis, etc., of the female.

2. They should be prepared by rolling swabs of secretion on glass slides (not rubbed on) with care not to pass the swab twice over the same surface.

3. Pus may be secured by massage of the urethra, the Bartholin glands, and from the vagina. In adults with chronic gonorrhea, it is particularly advisable to secure secretions from on and about the cervix with the aid of a vaginal speculum. Vaginal douches should be omitted for at least several hours before examination.

4. Several smears should be prepared and properly labeled. They should be neither too thick nor too thin. If the secretions are scanty, smears the size of pennies are sufficiently large. *They should be allowed to dry in the air*; the filthy practice of covering a thick wet smear of vaginal secretion with another slide is strongly condemned.

5. Cultures may be prepared by streaking the secretions on plates of ascites agar, North gelatin agar, the Pelouze medium or blood agar. Or swabs may be washed out into small amounts of sterile ascites fluid furnished in small test tubes with sterile rubber stoppers and the ascites emulsion sent at once to the laboratory for the preparation of plates. In medicolegal cases, fresh smears for active spermatozoa and cultures are usually required and the physician should enlist the services of the bacteriologist for aid in the technic of preparing them.

6. In infants and young children smears of the external genitalia are sometimes insufficient and unsatisfactory, especially in chronic infections with scanty secretions.

It is advisable to obtain material from the vagina by means of sterile slender cotton swabs for either smears or cultures or both. In older children, especially in those who have been under treatment, a nasal bivalve speculum may be employed as a vaginal speculum without injury, as it is particularly important to secure secretions on or about the cervix as they may show the presence of gonococci when smears of the external genitalia do not.

*Vaginal washings* are sometimes serviceable in these cases and may be conducted as follows:

- (a) Place the child on its back with thighs spread apart.
- (b) Fill the vagina with 1:4000 bichloride of mercury in normal saline solution by means of a sterile bulb or syringe.
- (c) Recover the washing and transfer to a centrifuge tube. Repeat until 5 to 10 cc. of washings have been secured.
- (d) *Centrifuge as soon as possible* and prepare smears of the sediment to be stained and examined for gonococci.

### COLLECTION OF MATERIAL FOR EXAMINATION FOR TREPONEMA PALLIDUM

1. *Treponema pallidum* is best found by darkfield examination of fresh material. Stained smears are much less satisfactory.

2. Wet preparations for darkfield examination may be prepared in the physician's office, providing a microscopy can be done in the laboratory before motility of spirochetes is lost. Otherwise it is better to send the patient to a laboratory equipped for this work. Or the physician may collect a drop or two of secretion in a Wright capillary blood tube (Fig. 135). The ends should be sealed with vaselin (not with heat). In the laboratory preparations are made for darkfield examination.

3. *Treponema pallidum* may be found in chancres (genital and extragenital), mucous patches, condylomata and in some skin lesions as well as in swollen lymph

glands, although examinations for the organisms are practically confined to sores regarded as possible primary lesions or chancres.

The examination of lesions on the lips is quite reliable but when occurring within the mouth great care is required, since *Treponema microdentium* of the saliva is morphologically indistinguishable from *Treponema pallidum*.

4. Surface exudates should not be used, as the spirochetes are usually in the tissues. An effort should be made to secure tissue "juice" with as little blood as possible. All applications of antiseptics should be omitted for at least several hours before the examination is made.

5. Thin glass slides (free of scratches) and coverglasses are required; also sterile saline solution and usually an instrument like a scalpel or stiff platinum wire loop for securing tissue juice. Capillary pipets may be used for collection. At least two slides should be prepared. Place a drop of saline solution on each.

6. The lesion should be grasped between the thumb and forefinger (protected with rubber gloves or gauze) and squeezed (Fig. 136) to secure tissue juice which may be transferred to the saline on the slides with a sterilized platinum wire or loop. If this is not successful, the lesion may be gently scraped (while being squeezed to prevent bleeding), the material transferred to slides and mixed with the saline solution. A coverglass should be applied (being careful to avoid floating) and the darkfield examination made at once. Or smears may be made and allowed to dry in the air if a staining method (like that of Fontana) is to be em-

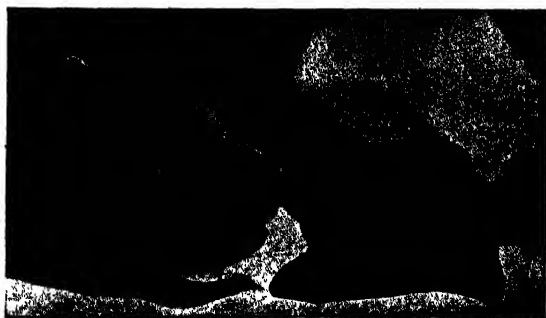


FIG. 136.—METHOD OF SECURING CHANCER MATERIAL  
(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, The Williams and Wilkins Co., Baltimore.)

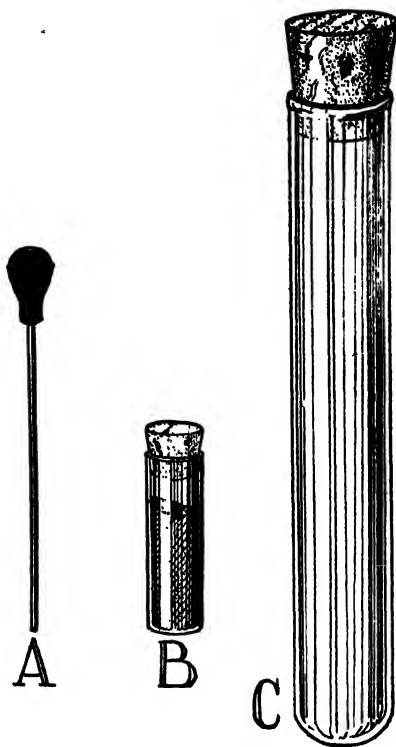


FIG. 135.—OUTFIT FOR THE COLLECTION OF CHANCER MATERIAL FOR DARKFIELD EXAMINATIONS

A, capillary tube with bulb for suction; B, vaseline for sealing capillary tube; C, test tube for carrying capillary tube.

ployed. If the sore is quite painful, one may first apply a few crystals of cocaine or a few drops of novocaine solution to anesthetize it.

7. Lymph gland material may be obtained by puncture, using a sterile 1 cc. syringe fitted with a No. 22 needle and injecting 0.5 to 1.0 cc. of sterile saline solution followed by aspiration and the preparation of slides with a few drops of the fluid.

### COLLECTION OF MATERIAL FOR EXAMINATION FOR HEMOPHILUS DUCREYI

1. Pus may be obtained by aspiration of a bubo with syringe and needle and inoculated into a medium of two parts agar mixed with one part of sterile human, dog, or rabbit blood; smears should be prepared at the same time.

2. An open ulcer may be painted with tincture of iodine and covered with sterile gauze; 24 hours later prepare smears and cultures of pus collecting under the dressing.

### COLLECTION OF MATERIAL FOR EXAMINATION FOR LEPROSY

1. Leprosi bacilli are usually present in the tissues of leprosy lesions in large numbers, especially in lepromatous leprosy. They may also be found in the nose and fauces.

2. The bacilli are chiefly in the fixed tissue cells, and it is usually necessary to scrape the lesions for proper material; little or no pain is produced (Fig. 137).

3. Place a drop of saline solution on each of several slides. Grasp the lesion and, while squeezing to prevent bleeding, scrape with a scalpel or a safety-razor blade and transfer the scraped-up tissue to the slides. Make spreads and allow them to dry in the air, to be followed by staining for acid-fast bacilli.

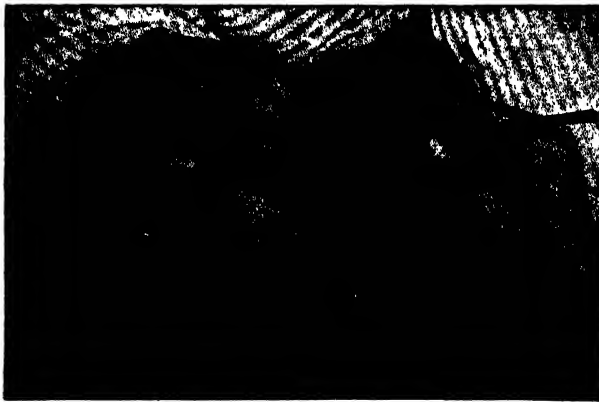


FIG. 137.—SECURING MATERIAL FROM A LEPROUS LESION

(From Bass and Johns, *Practical Clinical Laboratory Diagnosis*, The Williams and Wilkins Co., Baltimore.)

4. Secure material from the nose with a suitable instrument like the Freer sub-mucous elevator and prepare smears in the usual manner.

### COLLECTION OF MATERIAL FROM WOUNDS

1. All traumatic wounds, including war wounds, are invariably contaminated with bacteria, aerobic and anaerobic, from the soil, clothing, skin or air. Dead or devitalized tissues furnish excellent culture media for bacterial growth. If more than 6 hours elapse without definitive surgical aid, the contaminating microorganisms proliferate and produce infection. The immediate aim of treatment, to be followed by such surgical restoration as indicated, is the prevention or limitation of infection. Bacterio-

logical examinations of wound smears and cultures determine these procedures. Debridement is the first step by the surgeon in limiting infection, by removing all of the devitalized tissues and foreign substances which would provide a nidus of infection. By this procedure, bacteria are greatly diminished in number, but are not eradicated; at least most of the culture materials for bacterial growth are removed. Primary suture is not done except in quiet periods of warfare and in hospitals where the patient may be retained for careful observation; otherwise, wound suture may lead to enclosure, in an imperfectly debrided wound, of harmful microorganisms, especially of the gas gangrene group. Delayed primary suture may be done if the cultures, taken 18 to 48 hours after debridement, show no microorganisms; if hemolytic streptococci or staphylococci are present, suture is not considered. The presence of an additional microorganism per two fields (including a few anaerobes) does not contraindicate suture. Considerable numbers of microorganisms of any kind indicate delay of suture. Secondary suture is undertaken when the microorganisms, on two successive counts, are few and the culture has shown an absence of hemolytic streptococci and staphylococci.

2. No smears are taken while hemorrhage exists. Smears should not be taken within 2 hours after the application of Dakin's or other antiseptic solution. Smears need not be taken earlier than 12 hours after the infliction of the wound since up to that time few bacteria will be found. Smears are made of the wound secretions every other day, or daily, as the time of secondary closure occurs, in such a way that an appropriate estimate of the number of bacteria contained in the wound can be made.

3. Smears are taken with a platinum loop from different parts of the wound, choosing areas most likely to harbor bacteria, such as crevices, necrotic bone, foreign bodies, or deep sinuses; do not take from bleeding points, smooth muscle, or clean areas, and also avoid the skin adjacent to the wound. With a small platinum loop, small amounts of secretion are picked up and smeared on slides in such a way that about the same area is covered by the different loopfuls of secretions—with practice a uniformity of technic is attained to provide comparable bacteria counts. They should be allowed to dry before delivery to the laboratory.

4. When no bacteria can be found in smears, cultures of the wound secretions should be made on blood agar plates (without glucose) as it does not mean that the wound is completely sterile. If gangrene infection is suspected, a statement to this effect should accompany the specimen so that special anaerobic methods may be employed for *Cl. welchii*, *Cl. oedematis-maligni*, etc. If cultures are made at the bedside, blood agar plates are recommended, especially if mixed infection is suspected. Otherwise, blood agar slants on glucose-hormone broth may be used, suitable for the cultivation of streptococci. Plain agar may be used only in case of simple abscesses. Cultures are required particularly when the period of secondary closure approaches and especially if hemolytic streptococci or staphylococci have been found previously. Suture of a wound is not carried out if hemolytic streptococci or staphylococci of any kind are present; hence, frequent cultures upon blood agar plates are made during the progress of treatment. If the smears show a great many bacilli resembling the ordinary anaerobes, anaerobic cultures also may be made. However, because of the length of time required for working out the anaerobes in the laboratory, the surgeon is not concerned about this as a guide for his program of therapy.



**COLLECTION OF NECROPSY MATERIAL**

1. When bacteriological examinations are to be made in the course of a necropsy it is imperative to remove the material at the earliest possible time after death and at least within an hour or two in order to avoid the increase of secondary invaders and the postmortem invasion of the tissues with intestinal bacteria.

2. The body should not be embalmed if cultures are to be made, although smears and examinations for bacteria in sections of tissues may be made.

3. An area of the heart, liver or other organ or tissue to be cultured should be first seared with a cautery iron and then opened with a cautery or sterile knife and material obtained with a sterile pipet, cotton swab, stiff platinum wire or scalpel. Blocks of tissue may be removed, dipped into boiling water for surface sterilization and then cut into bits under aseptic precautions for cultivation in brain-hormone broth or similar enriched media.

**DISPOSAL OF MATERIAL SUBMITTED**

1. It is a good practice to retain specimens of pus, various secretions, spinal and other fluids, etc., for several days at least in case it is necessary or advisable to repeat the examinations. Cultures should be retained for a week or longer in case confirmatory tests are desired. It is also advisable to retain important smears properly labeled.

2. All specimens containing or likely to contain pathogenic organisms should be placed in a pail or pan and the latter autoclaved at the close of the day. Paper sputum cups should be burned.

3. It is a good practice to add a few cc. of 5 per cent phenol or tricresol to all discarded cultures (replacing the stoppers), including Petri plates.

4. Pipets used for handling infectious material should be placed in a jar, crock or pan of water containing phenol or tricresol for disinfection.

5. Boil all instruments and syringes immediately after use.

6. Wash the work table routinely with 10 per cent cresol or some other suitable antiseptic.

## METHODS FOR THE PREPARATION AND STERILIZATION OF GLASSWARE

**Principles.**—Test tubes, Petri dishes, and flasks should be made of good quality glass in order to (1) withstand repeated steam sterilization at 121° C. (approximately 15 pounds pressure) and hot air at 170° C. with the minimum of decomposition and (2) to contain the smallest amounts of free alkali so that there will be the minimum difference between the initial and final reactions of culture media. Strain-tested pyrex and nonsol glass are recommended because of their stability toward distilled water, and low coefficient of expansive and mechanical strength.

### SELECTION OF GLASSWARE

1. *Test tubes* should be of thicker walls than are used for chemical work, without lips in order to facilitate plugging and storage, and of such size as to fit in the wire test-tube racks now in common use. Three sizes are usually sufficient: 100 by 13 millimeters for slants and broth, as 5 cc. in each is sufficient and economical; 200 by 13 millimeters for giving a high column of medium with varying degrees of oxygen tension (low at the bottom and high at the top); 120 by 16 millimeters for carrying to 10 cc. or larger amounts of agar, gelatin, etc., for pouring Petri plates and for holding sterile swabs.

2. *Petri dishes* subjected to steam pressure sterilization should be of alkali-free glass and capable of standing repeated sterilization without corrosion (N-101-AF glass is recommended). Where hot air sterilization only is used, dishes of selected lime glass are sufficient, providing they are thoroughly dried before being placed in the sterilizer. Covers of white Coors porcelain are recommended; unglazed inside for providing sufficient absorbing surface for water of condensation, and glazed outside and at the sides for facilitating the removal of pencil markings. The 10-centimeter size is ordinarily employed.

3. *Erlenmeyer flasks* of pyrex or nonsol glass with vial mouths are recommended and three or four sizes are usually sufficient: 300 cc. capacity for blood cultures and 500 to 3000 cc. capacities for storage of culture media, etc.

4. *Smith fermentation tubes* (Fig. 138).

5. *Blake bottles* (Fig. 139) and *Kolle flasks* are commonly employed for mass cultures in the preparation of stock vaccines, bacterial antigens, etc., when large amounts are required.

6. *Microslides* should be of noncorrosive hard glass with ground edges, free of scratches and of the usual 3 by 1 inch size with a thickness of 1 to 2 millimeters. For darkfield work the slides should be carefully selected, free of scratches and within 1.45 to 1.55 millimeters in thickness.

7. *Micro coverglasses* should be of noncorrosive glass; soft white glass should not be used. Squares (22 millimeters) rectangles (22 by 36 millimeters), and round glasses (15 millimeters) may be recommended for routine work.

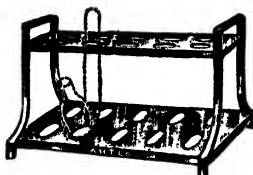


FIG. 138.—FERMENTATION TUBE SUPPORT



FIG. 139.—WIDE MOUTH RECTANGULAR BOTTLE

## CLEANING GLASSWARE

**Used Glassware.**—1. Glassware should be washed as soon after use as possible.

2. If glassware is contaminated, it should be autoclaved before being washed. After removing from the autoclave and while still warm, remove all cotton plugs and empty.

3. Place the glassware in warm water, add enough good quality soap to make plenty of suds and scrub well with a brush.

4. Rinse well in running water, preferably, rinsing once in tap water and twice in distilled water. Allow the glassware to drain and dry.

5. Tubes with paraffined stoppers should be *separately* cleaned because soiling with melted paraffin renders cleaning more difficult. After autoclaving and while still hot, remove the cotton stoppers, empty and immerse the paraffin-soiled glassware in hot water to which is added soap in fine shavings to make about a 5 per cent solution of the soap. Boil slowly for an hour, allow to cool somewhat and scrub well. If the paraffin is not removed, boil again in fresh soap solution, scrub well, rinse and allow to drain and dry.

*Pipets* should be placed, *immediately after using*, in a tall crock or cylinder full of 2 per cent lysol solution (for disinfection) with a layer of cotton on the bottom. To wash pipets, hold them in flowing tap water, or better, use a water suction pump attached to the faucet. Place the mouth end of the pipet in the rubber tube connected with the pump and the other end in a container of tap water and suck water through for about  $\frac{1}{4}$  minute; rinse by draining distilled water through the pipet, put aside to drain and dry.

*Slides and coverglasses* may be placed in 2 per cent lysol solution which in a week or so loosens Canada balsam; this solution also disinfects hanging drop and darkfield preparations.

1. Boil in soapy water or 10 per cent solution of chromic acid for 10 minutes.

2. Rinse in running water, drain and polish or keep the slides and coverglasses in 70 per cent alcohol.

**New Glassware.**—New test tubes, flasks, Petri dishes, pipets and other glassware are likely to contain free alkali and should, therefore, be allowed to stand for a few hours in 2 per cent hydrochloric acid. Rinse well with tap water, then treat as directed in steps 3 and 4 under used glassware in order to remove all traces of the hydrochloric acid.

Slides and coverglasses may be cleaned with alcohol and used directly or stored in 70 per cent alcohol until needed.

**Cloudy Glassware.**—When glassware (test tubes, Petri dishes, pipets, slides, coverglasses, etc.) retains cloudiness that cannot be removed by means of washing with soap, use potassium bichromate sulphuric acid cleaning solution, prepared as follows:

Commercial potassium bichromate ..... 60 gm.

Tap water ..... 300 cc.

Dissolve with the aid of heat, cool and add slowly  
with constant stirring:

Commercial sulphuric acid ..... 460 cc.

Fill test tubes, beakers, and flasks with cleaning solution and place pipets, slides, coverglasses, etc., in pans full of the solution and allow to remain 24 hours. To be followed by *thorough* rinsing in running water to remove all traces of cleaning fluid.

The fluid can be used repeatedly and when it appears to lose its strength more potassium bichromate and sulphuric acid should be added. It is, however, very corrosive and should not be used more frequently than necessary.

### PLUGGING TEST TUBES, FLASKS AND PIPETS

1. Test tubes may be plugged as follows: Using a good quality, long-fiber cotton batting which is nonabsorbent, cut strips  $1\frac{1}{2}$  to 2 inches in width; unfold the strips so that they are uniformly one layer thick; dip one end of a glass rod (about 3 inches long and 3 mm. in diameter) in water, then proceed to roll the flat strip of cotton about the moist glass rod until a plug of sufficient size is obtained; tear plug away from the remainder of the cotton, rolling a few times in the fingers and insert into a test tube. Remove the glass rod from the center of the cotton plug by turning a half turn in the opposite direction and withdrawing. The above method gives a well-formed plug which will not flatten out when withdrawn and reinserted into the test tubes. The cotton plugs for test tubes should be large and firm enough to exclude dust and germs, should project sufficiently beyond the tube for handling and be tight enough to permit the test tube to be lifted by the plug.

2. In the case of flasks, the strips of cotton should be cut wider and may be rolled with the fingers to the proper size.

3. In case it is desired to protect the interior of test tubes, flasks or other vessels from cotton fibers, a square of gauze or cheese cloth may be placed over the mouth of each vessel before inserting the cotton plug. This also allows support to the cotton plug in the case of flasks with large openings which require a large plug.

4. Added protection against contamination may be had by placing a piece of wrapping paper secured around the neck of the flask by means of cord.

5. The mouth ends of pipets should be plugged with a small pledget of cotton to protect the worker against accidental contamination of the mouth while pipeting microorganisms. The small bit of cotton may be inserted into the bore of the pipet by means of a piece of wire or a hairpin. The cotton should not extend beyond the end of the pipet in such a way as to prevent placing the finger firmly against the end. Any loose fibers can be burnt off by passing the ends of the pipets through a gas flame.

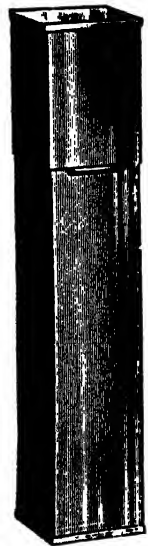


FIG. 140.—PIPET BOX

### STERILIZING GLASSWARE

1. Test tubes, Petri dishes, pipets and flasks should be *perfectly dry* before hot air sterilization, to prevent breakage and reduce decomposition. Do not use moist heat.

2. Petri dishes and pipets may be wrapped singly or in multiples with paper. Or they may be placed in special cans of copper or sheet iron for sterilization (Fig. 140).

3. Place in hot air sterilizer and *gradually* raise the temperature to about 170° C., which is sufficient for turning cotton and paper to a faint yellow color.

4. Avoid overheating to prevent charring of cotton and paper and the release of oils from cotton. Do not allow cotton plugs to touch the walls of the oven.

5. Heat for an hour at 170° C., and then allow the sterilizer to cool to at least 60° C. before opening the door, to prevent cracking by too sudden contraction of the glass.

6. While it is not imperative to sterilize test tubes and flasks before filling them with culture media, it is advisable to do so for ensuring better sterilization and for the purpose of molding the plugs for easier removal during bacteriological work.

7. When large amounts of glassware are to be sterilized, the use of a good thermo-regulating valve on the sterilizer together with a recording thermometer are recommended.

# METHODS FOR THE PREPARATION OF CULTURE MEDIA

## PRINCIPLES

1. Culture media are artificial foods for bacteria, containing soluble albumins, carbohydrates and other organic compounds as well as water and salts. Native proteins are probably not directly utilized and peptone is added to supply available nitrogen. Vitamin-like substances as well as other accessory substances present in blood, serum, ascitic fluid and fresh vegetable extracts help the growth of all pathogenic bacteria, especially streptococci, pneumococci, gonococci, *B. pertussis*, etc. Certain organisms such as the hemoglobinophilic *Hemophilus influenzae* require both the thermolabile V and the thermostable X factors in blood and vegetable tissues. Carbohydrates and especially glucose may be required. Dyes may be added either as indicators of metabolic activity or because of their selective inhibitory action on some bacteria, thereby aiding in isolation of others (notably the colon-typhoid group).

2. Therefore a very large number of media have been described for meeting special requirements. Practically all have as a general basis an infusion or watery extract of meat (usually beef or veal).

3. Heating for sterilization and filtering for clearing affects meat infusion deleteriously, and lost nutritive material is replaced in part by the addition of peptone, which is water-soluble and not precipitated by boiling; also by adding blood, serum, etc. Beef extract is generally inferior to fresh meat infusion because of the loss of nutritive substances due to prolonged heating in manufacture. It is, however, useful for the cultivation of the more hardy organisms and especially convenient for small laboratories.

4. The method of sterilization of culture media is, therefore, an important matter. Autoclaving may destroy nutritive principles. Fractional sterilization in an Arnold sterilizer is frequently to be preferred.

5. Most pathogenic bacteria are quite susceptible to acids and alkalis and grow best in media near neutrality or slightly alkaline or acid to it. The meat bases are more or less acid and usually require the addition of an alkali (sodium hydroxide). Culture media tend to become more acid during sterilization because of hydrolysis of some of the constituents. On the other hand, they may become more alkaline because of alkali derived from glass, especially if cheap, soluble glass containers are employed.

6. Culture media may be (a) fluid (broth, milk, peptone water, etc.); (b) solid (agar, gelatin, coagulated serum or egg, etc.), or (c) semisolid (broth with small amounts of agar, gelatin or coagulated serum); the last are especially useful for carrying stock cultures.

7. The most important principles involved in their preparation are: (a) To secure the maximum amounts of growth-stimulating substances, and (b) for this purpose to adjust the final reaction to the optimum pH for the organisms to be cultivated; (c) to use the minimum degree of filtration; (d) to use the minimum amount of heat for sterilization.

## DEHYDRATED OR POWDERED CULTURE MEDIA

Dehydrated culture media have been marketed for a number of years by the Digestive Ferments Company (Difco), Detroit, Michigan, and the Baltimore Bio-

logical Laboratory, Baltimore, Maryland. These types of media are often very satisfactory and are especially to be recommended to small laboratories where a trained mediamaker is not available. In larger laboratories the cost of such prepared media often becomes considerably higher than home-made media. For new methods of cultivation, isolation, or description of bacteria, only media whose composition and method of preparation are fully known or described should be used. Prepared powdered media may be used for routine work only when they can be shown to behave similarly to the original media described for the purpose.

### BASIC CONSTITUENTS OF CULTURE MEDIA

**Peptones.**—Peptones are peptic or tryptic digests of various proteins such as casein or various animal tissues. *Peptic digests* are usually made by adding minced animal stomachs to the ground protein in an acid medium. The temperature is generally kept at about 50° C. and the process of digestion controlled by making tests from time to time for various amino acids. The biuret test and the test for tryptophane are commonly used. *Tryptic digests* are made in slightly alkaline solution using pancreatic extract as a source of trypsin. If the digestion is carried out for more than 5 or 6 hours some preservative such as chloroform or toluol must be added. It is not always easy to decide what peptone to use for a particular purpose. Generally a considerable amount of comparative work must be done.

**Meat Extract.**—Commercial meat extract is generally made from so-called “soup liquor” obtained from the pre-cooking of meat intended for canning. The meat for canning is placed in large iron baskets and suspended in tanks of cold water. The whole is then heated with steam for 30 to 40 minutes. The liquor from this process is evaporated on vacuum pans at a temperature around 160° F. for some 4 hours. The concentrated extract is further evaporated in a kettle for some 8 hours or until the water content is down to about 22 per cent. During this process the extract turns dark brown and all the sugar and gelatin in the original juice are destroyed. The exact processes used by various companies are more or less commercial secrets.

**Agar-Agar.**—Agar-agar is obtained from certain types of “seaweed” found along the Pacific coasts. The greatest part of the agar supply comes from Japan, while a limited quantity is produced in California. The agar is extracted from the seaweed by boiling water. The resulting agar solution is allowed to gel, cut into strips and frozen. The frozen strips of agar are then allowed to thaw out, the agar dried, and bleached in the sun. Much agar is sold for bacteriological purposes in the original strip form cut into 8 to 10 inch lengths. The quality of agar used by bacteriologists has undergone considerable improvement during the last few years. It is now possible to obtain agar which is ground to any desired fineness and which gives a perfectly clear solution in water. Powdered agar needs no preliminary soaking in cold water and goes into solution after only 1 to 2 minutes of cooking.

For making streak plates concentrations of agar from 1.5 to 2 per cent should be used. For pour plates the best concentration is 1.2 to 1.5 per cent. Semisolid agar media generally contains about 0.3 per cent agar.

Due to the greater ease with which it goes into solution and the saving in time and effort by eliminating the necessity for filtration it is recommended that only powdered agar giving a clear solution in water be used.

**Gelatin.**—Gelatin is a protein obtained from bones, hornpiths, and hidestock. The bones are not used raw, but are first subjected to a chemical process which produces what is known as osseine, which is the organic substance of bones. The raw bones are washed, crushed, and then degreased by means of benzine or carbon tetrachloride. The mineral substances of the bones (mostly phosphate) are then extracted by diluted acid, usually hydrochloric or sulphurous, or a combination of these two. A valuable by-product, acid phosphate, is thus produced. The material left after extraction of the fat and mineral substances is called *osseine*. The osseine is subjected to various treatments of washing, alkali treatments to destroy the last traces of fat, more washing, acid treatment to destroy the alkali, and finally washing to obtain a neutral product. The material is then placed in extraction tanks where the gelatin is extracted by means of warm water (130 to 200° F.). The thin gelatin solution (4 to 6 per cent) is drawn off from the extractor and clarified by filtration and settling. For making sheet gelatin the dilute solution is spread on drums or endless belts on which it is chilled, scraped off and placed on cotton or wire netting to dry. For making powdered gelatin the solution is generally concentrated to 10 to 20 per cent and hardened in molds. It is then cut into thin pieces and dried on cotton or wire netting. The sheets or pieces of gelatin are dried by warm air, generally at from 85° to 105° F. There is some variation in gelatin made by different processes. The most important from a bacteriological standpoint is the gelling strength and clarity. The temperature and strength of acid used in the manufacture is probably responsible for any loss of gelling strength. Gelatin media should not be heated more than is necessary.

## METHODS FOR DETERMINING AND ADJUSTING THE REACTION OF CULTURE MEDIA

**Principles.**—1. The chemical reaction of the medium exerts a marked influence upon the growth of bacteria. It is adjusted after all ingredients are dissolved by adding sufficient sodium hydroxide solution to overcome the acidity of the meat and other substances used. Most media, particularly those containing sugars, become slightly more acid during sterilization with heat. Under the circumstances it is advisable when great accuracy is desired, to make a check titration upon a 10 cc. portion of the finished and sterilized medium which has been kept out for this purpose. The reaction is best adjusted to the proper pH by a colorimeter method.

2. In the pH scale, acid reactions scale lower than 7 and alkaline reactions scale above 7.

3. The pH of culture media is not increased or decreased in direct proportion to the amount of acid or alkali added. This is due to the action of buffer substances present in the media.

4. Buffers are substances which tend to inhibit a change in the pH when acid or alkali is added to a solution. Such substances as peptone, meat extracts, and phosphates have this inhibitory quality. Knowing the pH of a solution, it is not possible to figure mathematically the exact amount of acid or alkali to add to obtain a certain lower or higher pH, because of the action of these buffers.

5. Indicators are substances which when added to a solution assume a definite color at a particular pH. One indicator is useful only for a certain limited range of the pH scale. This is from the pH at which color begins to change to the pH where



the color change is complete, *e.g.*, phenol red gives a yellow color in solutions with low pH (acid side); at a pH 6.8 it begins to change to a pale pink which is intensified until a red is assumed at a pH 8.4 (alkaline side) and above.

6. It is obvious that several indicators are required to cover the entire pH range. Those commonly used are:

Methyl red	=	red to yellow	=	pH 4.4 to 6.0
Bromcresol purple	=	yellow to purple	=	pH 5.2 to 6.8
Bromthymol blue	=	yellow to blue	=	pH 6.0 to 7.6
Phenol red	=	yellow to red	=	pH 6.8 to 8.4

The bromcresol purple and bromthymol blue are used in 0.04 per cent; phenol red in 0.02 per cent solutions in 95 per cent alcohol.

7. Bromthymol blue is mostly employed since it gives a range of pH 6 to pH 7.6. With this indicator the color is yellow at pH 6; yellowish-green from 6.2 to 7; at pH 7 bluish-green which deepens to bright blue at 7.6.

8. The optimum pH for the more important bacteria, as determined by Fennel and Fisher, is as follows:

Pneumococcus	7.8
Streptococcus	7.6 to 7.8
Meningococcus	7.6
Gonococcus	7.5 to 7.6
Bacillus typhosus and B. paratyphosus	6.2 to 7.2
Bacillus dysenteriae	6.3 to 7.8
Bacillus influenzae	7.8 to 8.0

**Preparation of Indicator Solutions.**—*Bromcresol purple*, (0.2 per cent aq. sol.).—Dissolve 0.2 gm. bromcresol purple in a minimum amount of ethyl alcohol. Make up to 100 cc. with distilled water. *To be added to culture media as required.*

*Bromcresol purple*, (0.04 per cent alc. sol.).—Dissolve 0.1 gm. of bromcresol purple in 250 cc. of 95 per cent ethyl alcohol. *To be used as indicator for determining pH of media.*

*Bromthymol blue*, (0.2 per cent alc. sol.).—Dissolve 0.2 gm. of bromthymol blue in 100 cc. of ethyl alcohol. *To be added to culture media as required.*

*Bromthymol blue*, (0.04 per cent alc. sol.).—Dissolve 0.1 gm. of bromthymol blue in 100 cc. of 95 per cent ethyl alcohol. *To be used as indicator for determining pH of media.*

*Methyl red*, (0.02 per cent alc. sol.).—Dissolve 0.1 gm. of methyl red in 500 cc. of 95 per cent ethyl alcohol. *To be used as indicator for determining pH of media.*

*Neutral red*, (0.1 per cent sol.).—Dissolve 0.1 gm. of neutral red in 70 cc. of ethyl alcohol and dilute to 100 cc. with distilled water. *For use in culture media as required.*

*Phenol red*, (0.2 per cent stock sol.).—Dissolve 0.2 gm. of phenol red in 56.4 cc. of N/100 NaOH and dilute to 100 cc. with 95 per cent ethyl alcohol. *For use in culture media as required.*

*Phenol red*, (0.02 per cent sol.).—Dilute 10 cc. of the above stock solution to 100 cc. with 95 per cent alcohol. *Used as indicator in determining pH of media.*

*Andrade's Indicator.*—Dissolve 0.5 cc. acid fuchsin in 100 cc. of distilled water.

Add N/1 NaOH until color changes to pink, then to brownish red and finally to yellow (usually about 17 cc.). Shake the reagent after each addition of the alkali. The indicator is colorless at pH 7.2. *For use in culture media as required.*

**COLOR CHANGES OF THE INDICATORS OF CLARK, LUBS AND COHEN**

Indicator	Molecular Weight	Concentration Recommended *	Full Acid Color	Full Alkaline Color	Sensitive Range	pK †
Metacresol purple (acid range)	382	0.04	Red	Yellow	1.2-2.8	1.5
Thymol blue (acid range) . . . .	466	0.04	Red	Yellow	1.2-2.8	1.5
Bromphenol blue . . . . .		0.04	Yellow	Blue	3.0-4.6	4.1
Bromchlor phenol blue . . . . .	581	0.04	Yellow	Blue	3.0-4.6	4.0
Bromcresol green . . . . .	698	0.04	Yellow	Blue	3.8-5.4	4.7
Chlorcresol green . . . . .	520	0.04	Yellow	Blue	4.0-5.6	4.8
Methyl red . . . . .	269	0.02	Red	Yellow	4.4-6.0	5.1
Chlorphenol red . . . . .	423	0.04	Yellow	Red	4.8-6.4	6.0
Bromphenol red . . . . .	512	0.04	Yellow	Purple	5.2-6.8	6.2
Bromcresol purple . . . . .	540	0.04	Yellow	Red	5.2-6.8	6.3
Bromthymol blue . . . . .	624	0.04	Yellow	Blue	6.0-7.6	7.0
Phenol red . . . . .	354	0.02	Yellow	Red	6.8-8.4	7.9
Cresol red . . . . .	382	0.02	Yellow	Red	7.2-8.8	8.3
Metacresol purple (alkaline range) . . . . .	382	0.04	Yellow	Purple	7.4-9.0	8.3
Thymol blue (alkaline range) . . . .	466	0.04	Yellow	Blue	8.0-9.6	8.9
Cresolphthalein . . . . .	346	0.04	Colorless	Red	8.2-9.8	9.4
Phenolphthalein . . . . .	318	0.04	Colorless	Red	8.3-10.0	9.7

\* In 95% ethyl alcohol.

† Approximate invert logarithm of apparent dissociation constant.

Gillespie recommends an alcoholic solution only in the case of methyl red; for other indicators, it is recommended that the color acids be converted into sodium salts by preparing neutralized aqueous solutions (for technic see Vol. VI, No. 3, Aug. 1938 on Pure Culture Study of Bacteria edited by the Committee of the Society of American Bacteriologists).

**Colorimetric Methods.**—1. Sets of standard color tubes covering any pH range can be purchased ready prepared together with empty tubes of the same diameter (Fig. 141). Otherwise color standards of the indicator can be prepared in standard buffer solutions (Sørensen) to represent the shade of color obtained with a particular indicator at various points in the pH scale as follows:

2. Prepare a M/15 molecular acid or primary potassium phosphate solution by dissolving 9.078 gm. pure crystalline  $\text{KH}_2\text{PO}_4$  in 1000 cc. of freshly distilled ammonia-free water.

3. Prepare a M/15 molecular alkaline or secondary sodium phosphate by dissolving 11.876 gm. of pure crystallized  $\text{Na}_2\text{HPO}_4 \cdot 12(\text{H}_2\text{O})$ , which has been exposed to the air for 10 days to 2 weeks during which 10 molecules of water are given off, in 1000 cc. of freshly distilled ammonia-free water.

4. To make standard solutions of different pH concentrations in a graduated series mix the two phosphate solutions in the following proportions:

pH	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potass. phosphate, cc.	73	63	51	37	32	27	23	19	15.8	13.2	11	8.8	5.6	3.2	2.0
Secondary sod. phosphate, cc.	27	37	49	63	68	73	77	81	84.2	86.8	89	91.2	94.4	96.8	98.0

5. Place 2 cc. of the medium to be tested and adjusted in each of 2 test tubes. Add 8 cc. of freshly distilled water to each. If the water has been exposed to air for some time before use or is not freshly distilled, it should be boiled and allowed to cool to below 40° C. just before use. If agar is to be tested it can be measured while liquid and diluted with warm water (not over 40° C.) to prevent solidification.

6. To one of the tubes containing diluted medium, add the same amount of indicator as used in the standard tubes (ordinarily 0.25 to 0.5 cc.). Place the tube in the right front hole of the comparator block (Fig. 142).



FIG. 141.—LAMOTTE HYDROGEN ION SET



FIG. 142.—CLARK COLOR COMPARATOR

7. The other tube of diluted medium is placed in the left front hole of the comparator block.

8. Fill a third tube with distilled water and place in the right back hole of the comparator block, which is therefore behind the tube containing medium plus indicator.

9. From the set of standard color tubes select one which appears to match the tube of medium plus indicator. Place it in the left back hole, which is in back of the tube containing medium only. Compare the colors by viewing through the observation holes of the comparator block with either a daylight lamp or window furnishing light from the back of the block.

If the colors do not match, select another standard tube either lighter or more intense in color as may be indicated, and again examine.

Repeat this procedure until the tube is found which matches. The pH value marked on the tube which matches indicates the pH of medium.

10. To change the pH of a medium to another point in the pH scale, place the standard tube marked with the pH desired in the left-hand back hole and allow the other three tubes to remain in the same positions.

11. Slowly and carefully add N/20 solution of sodium hydroxide drop by drop to the tube containing medium and indicator until the color matches the color standard tube of the desired pH. Note the amount of N/20 solution used. The addition should be made from a buret or pipet and carefully measured. It is necessary to mix at intervals during the addition of the sodium hydroxide, especially when approaching the end-point, in order to avoid adding an excess.

12. To adjust the bulk of medium it is necessary to add to it sodium hydroxide in the same proportion as used to adjust the sample tested.

Suppose there are 2000 cc. of medium to be adjusted to a pH 7.6, and that it required 0.3 of N/20 sodium hydroxide to bring the tube containing 2 cc. medium and indicator to the same color as the standard tube marked pH 7.6. It would therefore require 300 cc. of N/20 sodium hydroxide to adjust 2000 cc. of medium. The addition of this amount of N/20 would increase the volume too much, so it is better to add one-twentieth of this amount or 15 cc. of a N/1 solution of sodium hydroxide.

The following is a simple method of calculation. Let *A* equal the amount of N/20 required to adjust 2 cc. of the medium to the proper pH, and *B* the number of cc. in the bulk of medium to be adjusted.

$$\frac{A \times B}{40} = \text{cc. of N/1 sodium hydroxide to add to bulk of medium}$$

or

$$A \times 25 = \text{cc. of N/1 sodium hydroxide to add to each liter of medium}$$

**Colored Glass Standards.**—A convenient type of pH colorimeter for large laboratories is the Hellige model (Fig. 143) containing permanent color standards as glass disks. The proper disk is inserted in the box and 10 cc. of medium added to the tubes, one of which contains the indicator. A wide range of pH values may be determined rapidly by using the appropriate disk. A standard solution of indicator must be used.

**Gillespie Standards.**—The method of Gillespie is perhaps the most reliable of the various colorimetric methods in that no standard solutions of any kind are required. The color corresponding to any desired pH is obtained by using a set of two tubes, one of which has 20 cc. of dilute acid and the other 20 cc. of dilute alkali.

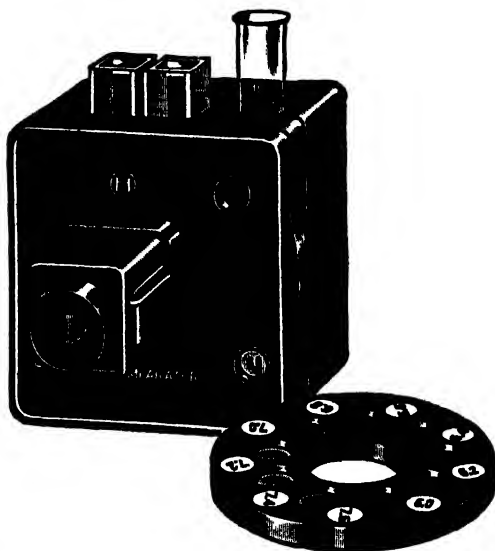


FIG. 143.—HELIGE COLORIMETER

A certain number of drops of indicator is added to the acid and alkaline tubes. The ratio of the amount of indicator in the acid and alkaline tubes determines the shade of color and hence the pH, when looking through both tubes. The indicator solution need not be of any exact concentration since the same indicator is added to both the standard and the unknown. In the following table is given the respective number of drops of various indicators to be added to the acid and alkaline tubes to obtain any desired pH. In this table is shown the pH obtained when various amounts of indicator are added to the alkaline and acid tubes:

Drop-Ratio Alkali: Acid	pH M.R.	pH B.C.P.	pH B.T.B.	pH P.R.	pH Thymol Blue
2—18	4.05	5.30	6.15	6.75	7.85
3—17	4.25	5.50	6.35	6.95	8.02
4—16	4.40	5.70	6.50	7.10	8.20
5—15	4.50	5.80	6.60	7.20	8.30
6—14	4.60	5.90	6.70	7.30	8.40
7—13	4.70	6.00	6.80	7.40	8.50
8—12	4.80	6.10	6.90	7.50	8.60
9—11	4.90	6.20	7.00	7.60	8.70
10—10	5.00	6.30	7.10	7.70	8.80
11—9	5.10	6.40	7.20	7.80	8.90
12—8	5.20	6.50	7.30	7.90	9.00
13—7	5.30	6.60	7.40	8.00	9.10
14—6	5.40	6.70	7.50	8.10	9.20
15—5	5.50	6.80	7.60	8.20	9.30
16—4	5.60	6.90	7.70	8.30	9.40
17—3	5.75	7.00	7.85	8.45	9.50
18—2	5.95	7.20	8.05	8.65	9.75

A comparator block with 2 sets of 3 holes in series is used. These are sold by laboratory supply houses as the Gillespie Comparator Block. Large tubes of uniform diameter should be selected for use and graduated to 10 and 20 cc. for convenience. Only 4 to 5 drops of N/10 acid or alkali should be added to the standard tubes since an excess of acid or alkali may change the color of the indicator. The medium to be titrated is diluted as desired and 10 cc. placed in each of 2 tubes. To one of these is added 10 drops of the indicator and this is placed in the block in series with 2 tubes of water. The tube of medium without indicator is placed in series with the color standard.

**Electrometric Methods of Determining pH of Media.**—Electrometric methods are the most fundamental methods of determining the reaction and for the titration of culture media. All colorimetric methods must be standardized electrometrically. While electrometric methods are more fundamental and accurate they also require greater care, more controls, and are generally too cumbersome for the small laboratory to use. This is especially true of the most fundamental method of all which is the use of the hydrogen electrode. By means of this electrode a direct measurement is made of the potential, or effective concentration, or hydrogen ions. All other methods, whether electrometric or colorimetric must be standardized against the hydrogen electrode.

The glass electrode and the quinhydrone electrode are simpler to use than the hydrogen electrode, but have a limited usefulness. For titrating or determining the

reaction of highly colored liquids, checking on colorimetric methods, etc. these methods are useful and sometimes indispensable.

For a detailed discussion of these methods the reader is referred to such texts as Clark's *Determination of Hydrogen Ions*. For the average bacteriological laboratory the colorimetric methods are sufficiently accurate and to be preferred.

**Titrimetric Phenolphthalein Method.**—The color changes of phenolphthalein are:

- (a) Colorless: medium is acid.
- (b) Faint pink: medium is neutral to phenolphthalein, but actually on the alkaline side of true neutrality equal to pH 8.2 to pH 10.0.
- (c) Red: medium is alkaline.

The plus sign is used for denoting acidity and the minus sign for alkalinity, according to Fuller's method.

1. The materials required are (Fig. 144):

- (a) A buret (B) held in a clamp on a ring stand (A)
- (b) Casserole (C)
- (c) Glass stirring rod
- (d) Normal (N/1) and twentieth normal (N/20) sodium hydroxide solutions
- (e) Indicator: dissolve 0.5 gram phenolphthalein in 100 cc. of 50 per cent alcohol (0.5 per cent solution)

2. Put 45 cc. of *freshly boiled* and cooled distilled water and 5 cc. of medium in the casserole. If agar is being titrated, have the water at about 40° C. to keep the medium fluid.

3. Add 1 cc. of indicator solution. As a general rule, there is no color change, indicating that the medium is acid.

4. Place N/20 sodium hydroxide solution in the buret and record the reading or level.

5. Add small amounts of the sodium hydroxide solution to the medium, stirring briskly after each addition.

6. Stop at the *first faint pink tinge*.

7. Read the buret and record.

8. Subtract this reading from the first to give the amount of sodium hydroxide required for 5 cc. of medium. Example: 2.1 cc. N/20 sodium hydroxide used. The medium has an acid reaction recorded as +2.1 according to the Fuller method.

9. If it is desired to render the bulk of the medium neutral to phenolphthalein, proceed as per the following example:

5 cc. require 2.1 cc. of N/20 sodium hydroxide

100 cc. require 42.0 cc. of N/20 or 2.1 cc. of N/1 sodium hydroxide

1000 cc. require 21 cc. of N/1 sodium hydroxide

10. A shorter method of calculation is as follows.

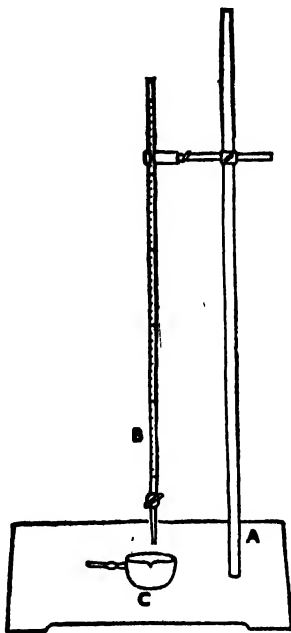


FIG. 144.—OUTFIT FOR PHENOLPHTHALEIN TITRATION

(From Park, Williams and Krumwiede, *Pathogenic Microorganisms*, Lea and Febiger, Philadelphia.)

Let *A* equal the amount of N/20 required for 5 cc. of medium and *B* the bulk of the medium:

$$\frac{A \times B}{100} = \text{cc. of N/1 sodium hydroxide required for rendering the bulk of medium neutral to phenolphthalein}$$

11. If it is desired to have the medium slightly acid to phenolphthalein, for example, + 0.1, add 20 cc. of N/1 sodium hydroxide instead of 21 cc.; if an endpoint of + 0.2 is desired, add 19 cc. of N/1, etc.

12. This method, however, is only approximately correct because the buffer substances present in the medium combine with some of the sodium hydroxide so that one does not know exactly how much acid has been neutralized nor the actual acidity of the medium. For this reason the hydrogen ion method of titration is to be preferred.

13. Few media are naturally alkaline to phenolphthalein, that is, yield a red color upon the addition of this indicator. In this event titrate with N/20 solution of hydrochloric acid to determine the quantity required for reducing to neutrality (faint pink color), and calculate as above. If, however, a medium has been rendered too alkaline by the addition of too much sodium hydroxide, it is better to discard it than to reduce the alkalinity with hydrochloric acid, as this generally renders the medium unsatisfactory for the cultivation of sensitive bacteria.

14. The above is the *room temperature* or *standard method* to be applied to the titration of culture media brought to the boiling point in preparation before titration. If, however, the medium has been heated to only about 50° C. for dissolving the ingredients, it is necessary to boil the mixture of 5 cc. in 45 cc. of distilled water for 1 minute before the titration is conducted (the *boiling method*) to allow for the chemical changes to which the bulk of medium will be subjected during sterilization.

**Methods of Determining Reaction of Bacterial Cultures.**—To determine the reaction of bacterial cultures a special technic is not necessarily required. Certain factors, however, make it convenient and often advisable to use a special technic. This is especially true where the volume of the culture is small and repeated determinations have to be made. In such cases the sample for each test is very small and must be taken with aseptic precaution. In dealing with cultures of pathogenic bacteria the tubes must be sterilized upon completion of the test and this is often inconvenient with the usual technic.

The simplest technic for determining the reaction of bacterial cultures seems to be that of Brown. A large platinum loop or Pasteur pipet is used to transfer a small quantity of the culture to a little glass cup filled with distilled water plus a drop of the desired indicator. Similar cups are filled with buffer solutions at various pHs and a drop of indicator added to each. The pH of the culture is determined by comparing the color in the cup with medium to the colors in the cups with buffers. By placing the cups on a plate of milk glass very accurate pH determinations can be made. The indicators are best made up in 60 per cent alcohol solution. The indicator solutions should be titrated to the midpoint of their color range. The complete apparatus with directions is made by the La Motte Chemical Co. and may be bought from laboratory supply houses.

**Methods of Using Indicators in Culture Media for Showing Changes in Reaction Due to Bacteria.**—To determine the reaction of bacterial cultures, espe-

cially in carbohydrate media for fermentation studies, indicators may be added to the media before sterilization. These indicators must not be toxic and should be relatively stable. Indicators can generally not be added to anaerobic culture media because they are reduced.

(a) For media with a pH from 5.2 to 6.8 a good indicator is bromcresol purple.

(b) For media with a pH from 6.6 to 7.6 the best indicator seems to be bromthymol blue.

(c) For media with a pH from 7.1 to 8.4 the best indicator is phenol red.

## CLEARING AND FILTRATION OF CULTURE MEDIA

1. Culture media should be clear, but too fine filtration may remove growth-stimulating substances.

2. Large particles may be removed by sedimentation as in the preparation of "hormone" or "vitamin" media: After heating, allow broth media to stand overnight in the icebox and decant or pipet off the supernatant fluid next day without disturbing the sediment. Allow agar to stand overnight in a *straight side* container; turn out, trim off and discard the sediment. The agar may also be allowed to sediment in a pot and may be removed with a spoon. The sediment on the bottom layer is trimmed off with a knife. The finer particles remain in suspension and no further attempt is made at clearing except filtration through a fine wire mesh.

3. Clearing is also accomplished by coagulation of albumins during heating (boiling, Arnold sterilizer or autoclave) with enmeshment of particles, or by adding an egg to each liter of medium to furnish coagulable albumin. Mix one egg in a pan with an equal amount of water and add to the medium. If dried egg albumin is used, dissolve 10 grams in 20 cc. of water and add to the medium. The medium is then heated in Arnold sterilizer for 45 to 60 minutes or autoclaved for 30 minutes; ordinary boiling is not as good. Clearing with egg, however, should be avoided whenever possible because of the possibility of adding sulphur and fermentable substances tending to interfere with the growth of some organisms.

4. For the filtration of broths and similar fluid media various grades of fine and coarse filter paper may be employed with a fluted funnel (Fig. 145) or a plain funnel with a wire rack inside.

5. Agar and gelatin media must be filtered while hot and fluid. Place a small square of coarse wire netting in the funnel and cover the netting with a thin layer of absorbent cotton. Some pour boiling water through the cotton, but this is perhaps unnecessary and the water dilutes the medium. The agar is best poured through the dry cotton and filtered into flasks. By using preparations of agar which give clear solutions in water filtration of agar media can be sometimes omitted.

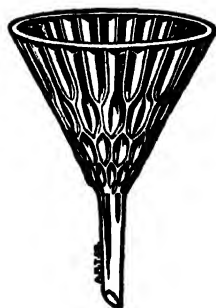


FIG. 145.—FLUTED GLASS FUNNEL FOR RAPID FILTRATION



### STERILIZATION OF CULTURE MEDIA WITH HEAT

1. Culture media may be sterilized by (a) heat (autoclave, Arnold sterilizer or water bath) or (b) filtration. Volatile disinfectants like chloroform may be used for the preservation of serum, ascites fluid, etc., but chemical disinfection has not as yet been perfected.

2. The minimum of heat should be used, as overheating may destroy growth-stimulating substances as well as caramelizing and hydrolizing sugars.

3. *Autoclaves* should be equipped with thermometers, as the temperature is more reliable than pounds of pressure. In general terms the equivalents are as follows:

5 pounds pressure.....	107.7° C. (226° F.)
10 pounds pressure.....	115.5° C. (240° F.)
15 pounds pressure.....	121.6° C. (250° F.)
20 pounds pressure.....	126.6° C. (260° F.)
25 pounds pressure....	130.5° C. (267° F.)
30 pounds pressure.....	133.5° C. (274° F.)

4. Two kinds of autoclaves are available: (a) the upright for small laboratories and (b) the horizontal for larger laboratories. The former is heated with gas, kerosene, electricity (special connection required) or flowing steam; the latter may be heated with gas or connected with a steam plant.

5. For media in test tubes, sterilization at approximately 121° C. for 15 to 20 minutes is sufficient; for media in bulk, 30 minutes are required.

6. With either autoclave allow time for agar to melt before timing the period of sterilization. Allow autoclave to cool before opening the door, as a sudden release of pressure may wet or blow the stoppers and crack glassware.

7. The *Arnold sterilizer* furnishes streaming steam at 100° C. and is especially recommended for routine use, as overheating is readily avoided and likewise caramelization of sugars. The Arnold has the further advantage that the volume of liquid re-

mains constant.

Make sure that there is plenty of water in the pan to avoid the possibility of boiling dry followed by the melting of soldered joints with danger of fire.

Media in test tubes should be heated for twenty minutes *after steam is produced*, and bulk media for 45 to 60 minutes on each of three days in succession (*fractional sterilization*), allowing extra time for the melting of agar. Allow the sterilizer to cool before removing the contents.

8. *Water bath* sterilization is adapted only for small amounts of serum, ascites fluid, tissue extracts, etc., to prevent coagulation. The material should be as free as possible from contamination. This method is valueless for killing spores. The water should be above the level of the fluid to be sterilized. The temperatures should be 60° C. for about 2 hours.



FIG. 146.—A WATER SUCTION PUMP

### STERILIZATION BY FILTRATION

1. Filtration methods are mainly employed for the removal of bacteria from serum, ascites and hydrocele fluids, tissue extracts, etc., that cannot be sterilized by heating at 60° C. (as when spores are present), for separating the cellular from the soluble products of bacterial growth and for sterilizing solutions of carbohydrates.

2. Berkefeld filters are usually made of diatomaceous earth of negative electrical charge and with pores small enough to hold back bacteria and spores (Berkefeld "W" = very fine; "N" = normal or medium and "V" = coarse for rapid filtration).

3. Suction or pressure must be provided, suction being provided by a suction pump attached to a faucet (Fig. 146).

4. For the filtration of large amounts, the Mandler (Fig. 147) and Seitz (Fig. 148) filters may be recommended. The larger sizes of these require suction or pressure pumps. The Haen is a new form employing a membrane.

5. The filter candle and all attachments with which the filtrate will come in contact must be sterile. The glassware may be sterilized in a hot air oven; the candle and rubber connections may be boiled for an hour or autoclaved.

6. New candles should be cleansed before use by passing through distilled water, followed by placing in cold water and boiling for 30 to 60 minutes. It is sometimes advisable to test for impermeability to bacteria with a broth culture of *B. prodigiosus*.

7. Paraffin, petrolatum, and other oils must be carefully avoided, as they tend to increase permeability to bacteria.

8. After use, candles should be cleansed by filtering through distilled water (to remove soluble and especially coagulable material) followed by sterilization by boiling (if infective material has been used) and a light scrubbing of the surface with a fine brush.

9. After continued use, candles become clogged (the average is approximately 10 filtrations) and must be discarded. They may be heated to a glow, but this tends to produce cracks and increase their permeability.

10. Before candle filtration the material should be first filtered through a fine filter paper or paper pulp to remove large particles and reduce clogging.

11. Filters of the Mandler or Berkefeld types may be tested for gross leaks by immersing them in water and connecting them to a source of air pressure. A readily controlled source of air pressure may be obtained by slowly running water from a tap into a large bottle provided with a rubber stopper. The inside of the bottle is connected through the stopper with a mercury manometer and with the filter to be tested. The pressure of the air in the bottle is gradually increased by the inflowing

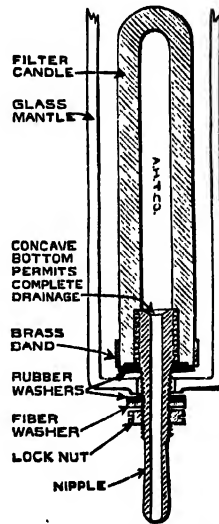


FIG. 147.—SECTIONAL VIEW OF MANDLER FILTER CYLINDER



FIG. 148.—SEITZ UHLENHUTH FILTER

water and the manometer reading taken at which air bubbles first come through the filter. Defective filters are readily noted by this means and a good idea is obtained as to the condition of the filter. A good Berkefeld N filter should not pass bubbles much below a pressure of 15 inches of mercury.

## FORMULAS AND DIRECTIONS FOR PREPARATION OF CULTURE MEDIA

**Principles.**—The preparation of bacteriological culture media is fundamental to all bacteriological work whether routine or research. Much of the care and skill which goes into the preparation of food for human consumption should go into the preparation of culture media for bacteria. Media rooms should not be located in a dark and dusty basement, as is often the case, but should be modeled on a modern kitchen. The media maker should be trained in the principles of physics and chemistry as well as bacteriology.

When directions for the preparation of media are carefully written, they should be followed to the letter. Only pure chemicals should be used except where specifically otherwise stated. All media should be prepared in scrupulously clean vessels preferably pyrex (or similar glass), enamel, or tinned copper. Aluminum vessels should not be used because it has been found that they impart a certain toxicity to the medium. The reaction should be adjusted carefully, generally to within 0.1 unit of the pH specified. Sterilization should be carefully controlled as to time and temperature. In using the autoclave, it is especially important that all air be removed. It must be kept in mind that air is heavier than steam and that air pockets develop in the bottom of the autoclave and not in the top. It is also well to keep in mind that a loss of volume always takes place in the autoclave.

Media sterilized in bulk should be stored in closed containers, preferably glass, unless intended for immediate use. Crown capped milk bottles are very convenient and inexpensive for storing media. New bottles must always be heat-treated by pouring them full of boiling water and autoclaving before they are used for media. Tubed media are generally best stored in the refrigerator to lessen evaporation. The cotton plugs may also be removed following sterilization and replaced by sterile rubber stoppers. A better way is to push the cotton plug into the tube flush with the top and cover the end of the tube with a rubber cap previously dipped in 5 per cent phenol solution. Paraffining the cotton plugs will also lessen evaporation. Concentrated sugar solutions can be kept from evaporating by means of a vaseline seal. By this means tubed media may be kept for many months following sterilization.

**Beef Extract Broth.**—For general cultivation of less fastidious bacteria. As basis for carbohydrate broths, agar, gelatin, etc.:

<i>Composition.</i> —Bacto-peptone .....	5.0 gm.
Beef extract .....	3.0 gm.
Sodium chloride (C.P.) .....	5.0 gm.
Distilled water .....	1000.0 cc.

*Preparation.*—Combine the ingredients and dissolve by heating. Adjust to pH 7.0 and boil for a few minutes. Add water to make 1000 cc. and filter through paper until clear. Dispense and autoclave at 121° C. for 20 minutes.

**Meat Infusion Broth.**—For general cultivation of bacteria and as a basis for preparation of agar, gelatin, etc. The technic preserves the "hormone" principle:

<i>Composition.</i> —Chopped lean beef or veal.....	500.0 gm.
Bacto-peptone .....	10.0 gm.
Sodium chloride (C.P.) .....	5.0 gm.
Distilled water .....	1000.0 cc.

*Preparation.*—Infuse the ground meat in the water over night at 4° to 6° C. For double strength infusion, use 1000 gm. to 1000 cc. of water. Skim off the fat. Heat to 45° C. and hold between 45° C. and 50° C. for 1 hour. Boil for ½ hour without stirring. Lift out the firm coagulum or put through a sieve (must not be of copper but glass wool is permissible). Add distilled water to make up to the original volume. Add the peptone and salt and apply heat until dissolved. Adjust the reaction to desired pH. Bring to boiling point and check the reaction. Do not filter but allow to stand until suspended particles have settled. Decant or siphon off the clear fluid. Dispense and autoclave at 121° C. for 15 minutes.

**Huntoon Hormone Broth (Modified).**—For the cultivation of fastidious bacteria:

<i>Composition.</i> —(A) Fresh ground beef heart. (Do not remove fat before grinding) .....	15 pounds
Eggs .....	9
Distilled water .....	10 liters

Stir the eggs in about 1 liter of water and then add to the meat and water. Stir occasionally while the mixture is slowly heated to 50° C.

(B) Peptone .....	150.0 gm.
Sodium chloride .....	37.5 gm.
Distilled water .....	5.0 liters
Bring to a boil and slowly add gelatin..	150.0 gm.

*Preparation.*—When A has reached 50° C., add B. Stir thoroughly and bring to a boil. Do not stir after the first mixing. Boil 10 minutes. Remove about 500 cc. of fluid and filter through paper; 50 cc. portions are placed in 100 cc. beakers. To each beaker is added an increasing amount of N/1 hydrochloric acid. Begin with 0.5 cc. and increase by 0.25 cc. to 1.5 cc. One of these amounts of hydrochloric acid will cause the maximum precipitate. An aliquot portion of hydrochloric acid is added to the main portion of the mixture. Boil 10 minutes. Strain through a colander. (*Do not filter through any organic material.*) Autoclave at 121° C. for 15 minutes. Pour into 6 liter glass jars and allow sedimentation to proceed for 24 hours at room temperature. The supernatant medium is siphoned off leaving the fat and sediment behind. The pH is adjusted to 8.0 in the cold with N/1 sodium hydroxide and the volume is made up to 15 liters after the addition of the sodium hydroxide. Bring to a boil and pour into glass jars. In 2 or 3 hours the flocculent precipitate which has formed settles out. The supernatant is siphoned off into large flasks and autoclaved at 121° C. for 15 minutes. It is advisable to store these flasks for several days so that if a precipitate forms after the autoclaving, the supernatant can again be siphoned off. Repeated autoclaving will not affect this medium. If this broth is properly made it will support a luxurious

growth of gonococcus or meningococcus without the addition of any growth promoting substances, such as ascitic fluid.

**Beef Infusion Broth with Proteose.**—A basic broth for the growth of hemolytic streptococci and preparation of blood agar:

<i>Composition.</i> —Group beef .....	500.0 gm.
Distilled water .....	1000.0 cc.
Peptone, Difco proteose .....	20.0 gm.
Sodium chloride (C.P.) .....	5.0 gm.

*Preparation.*—Mix the meat and water and infuse overnight at from 4° to 6° C. Strain through cheesecloth and press the juice from the meat in a meat press. Boil for 30 minutes, stirring occasionally. Strain through cheesecloth and filter through paper. Dissolve the peptone and salt in the filtrate. Make up to original volume with water. Adjust the reaction to pH 8.2. Autoclave at 121° C. Retitrate, adjust pH to 7.6 and boil for 3 minutes. Filter through paper until clear. Dispense and autoclave at 121° C. for 20 minutes.

**Cooked Meat Medium.**—For cultivation of anaerobes:

Cut 500 gms. of lean beef or veal heart into small cubes and cover with distilled water. Bring to a boil and simmer over a low flame for 1 hour. Strain off and set aside the fluid infusion. Pass the meat 3 times through a meat grinder and then break up the particles by rubbing them between the hands.

To the fluid infusion add sufficient distilled water to make 2 liters and mix it with the meat. Add N/1 sodium hydroxide until the reaction of the supernatant fluid is pH 8.0. Weigh the medium in a tared vessel. Autoclave for 15 minutes. Restore the weight with distilled water. Readjust the reaction to pH 8.0. Boil for 10 minutes. Restore the weight with water and readjust the reaction to pH 8.0. Boil again for 10 minutes. Restore the weight and if the reaction is more acid than pH 7.5, readjust to this reaction.

Distribute into tubes, keeping the mixture well stirred so that there may be a uniform deposit of meat particles in each tube. Cover with a layer of sterile vaseline and sterilize in the autoclave. Check the final reaction which should be about pH 7.1 and must not be acid. The repeated adjustment of reaction and boiling is necessary because the supernatant fluid contains little buffer whereas the meat particles slowly take up large amounts of alkali. The reaction of the mixture is difficult to stabilize.

**Brewer's Sodium Thioglycollate Broth.**—For the cultivation of anaerobes:

<i>Composition.</i> —Pork infusion solids .....	10.0 gm.
Peptone .....	10.0 gm.
Sodium chloride .....	5.0 gm.
Sodium thioglycollate .....	1.0 gm.
Agar .....	0.5 gm.
Dextrose .....	10.0 gm.
Methylene blue .....	0.2 gm.
Water .....	1000.0 cc.

*Preparation.*—Dissolve the solids with slow heating. Tube and autoclave at 121° C. for 20 minutes. Store at room temperature. Oxygen returns the color of the methylene blue and the aerobic portion (top) of the tube becomes green. If the color extends

over 1½ inch below the surface, re-autoclave the tubes, which again makes them strictly anaerobic.

**Douglas Tryptic Digest Broth.**—1. Mix 150 gm. of ground lean beef with 250 cc. of distilled water.

2. Heat to 80° C. and add 250 cc. of 0.8 per cent solution of anhydrous sodium carbonate.

3. Cool to 45° C., and add 5 cc. of Cole and Onslow's pancreatic extract prepared as follows:

Pig pancreas, fresh, fat-free, minced .....	500.0 gm.
Distilled water .....	1500.0 cc.
95 per cent alcohol .....	500.0 cc.

Mix the pancreas, water, and alcohol thoroughly; place the mixture in a large stoppered bottle; allow to stand at room temperature for 3 days; shake repeatedly.

4. Strain through gauze and filter through paper.

5. To each 100 cc. add 0.1 cc. of concentrated hydrochloric acid.

6. Filter and incubate at 37° C. for 6 hours, stirring frequently.

7. Add 40 cc. of normal hydrochloric acid and boil for 1 hour.

8. Cool and filter through paper.

9. Adjust to pH 7.6 to 7.9. Boil for 1 hour, and filter through paper after cooling to about 45° C.

10. Dispense and autoclave at 121° C. for 15 minutes.

**Buffered Meat Infusion Dextrose Broth.**—For the cultivation of streptococci and other fastidious organisms:

<i>Composition.</i> —Meat infusion broth .....	1000.0 cc.
Neopeptone .....	10.0 gm.
Sodium chloride (C.P.) .....	3.0 gm.
Dibasic sodium phosphate.....	2.0 gm.
Dextrose .....	1.0 gm.

*Preparation.*—Mix ingredients and heat to boiling. Adjust reaction to pH 8.3. Boil to throw down precipitate. Filter or decant. Dispense and autoclave at 121° C. for 20 minutes.

**Carbohydrate Broths for Aerobic Bacteria.**—*Preparation.*—Beef extract broth to which is added 5 or 10 gms. of the desired sugar per 1000 cc. and the indicator. The best general indicator is bromcresol purple or chlorphenol red (15 cc. of 0.2 per cent stock solution per 1000 cc.). If it is desired to use the medium at a pH of 7.2 to 7.5 the preferred indicator is phenol red (10 cc. of 0.2 per cent stock solution per 1000 cc.). Adjust to pH 7.4. Dispense and autoclave at 121° C. for 15 minutes or sterilize in the Arnold by the intermittent method.

**Hiss' Serum Water Inulin Medium.**—For differentiation between streptococci and pneumococci:

1. Mix 100 cc. of blood serum with 300 cc. of water.

2. Steam in the Arnold sterilizer for 15 minutes to destroy any diastase that may be present.

3. Adjust reaction to + 0.2 to + 0.8.

4. Add litmus or azolitmin solution to give a bluish-violet color.

5. Prepare a 10 per cent solution of inulin in water and sterilize in autoclave at 121° C. for 30 minutes.

6. To each 90 cc. of the heated serum-water add 10 cc. of the sterilized inulin solution.

7. Sterilize in the Arnold for 30 minutes on each of 3 days in succession.

**Starch Serum Broth Medium.**—For differentiation between the *mitis* and *gravis* types of diphtheria bacilli:

1. Prepare a 2 per cent aqueous solution of soluble starch and sterilize in the Arnold for 1 hour.

2. To 375 cc. of sterile infusion broth add 100 cc. of the sterile starch solution and 25 cc. of sterile human, horse or rabbit serum. Add bromcresol purple as indicator.

3. Place 5 cc. in sterile test tubes and incubate at 37° C. for 24 to 48 hours for sterility. Dunham tubes are not required because gas is not produced.

**Carbohydrate Broths for Clostridia (Reed and Orr).**—

<i>Composition.</i> —Bactopeptone or proteose peptone..	20.0 gm.
Sodium chloride .....	5.0 gm.
Sodium thioglycollate .....	1.0 gm.
Agar .....	1.0 gm.
Water .....	1000.0 cc.

*Preparation.*—Dissolve with gentle heating and autoclave at 121° C. for 30 minutes.

Prepare 10 per cent solutions of the desired carbohydrates in distilled water and sterilize by filtration. Add 1 cc. to each 100 cc. of the sugar-free base (1 per cent concentration). Dispense in test tubes. Fermentable carbohydrates are sufficiently broken down by the anaerobic bacilli in 24 hours to give an acid reaction.

Bromthymol blue is a satisfactory indicator, but since it tends to be reduced during the growth of anaerobes it is added at the end of the growth period and the reactions read at once. A 1 per cent alcoholic solution of the dye may be employed.

**Serum and Ascitic Broths.**—For the cultivation of fastidious aerobic bacteria:

These are prepared by adding aseptically 2 to 5 per cent of sterile blood serum or ascites fluid to sterile infusion or extract broth. Dispense in test tubes and incubate at 37° C. for 48 to 72 hours for sterility.

For fermentation studies add 1 cc. of a sterile 10 per cent solution of carbohydrate to 100 cc. of medium and dispense in tubes (1 per cent concentration). The carbohydrate solution may be sterilized in the Arnold, but sterilization by filtration is preferred.

**Rosenow Glucose Brain Broth.**—For the isolation of fastidious bacteria, especially streptococci and microaerophilic bacteria:

<i>Composition.</i> —Meat infusion broth .....	1000.0 cc.
Bacto-peptone .....	5.0 gm.
Sodium chloride (C.P.).....	8.0 gm.
Glucose .....	2.0 gm.
Andrade's indicator .....	10.0 cc.

*Preparation.*—Dissolve peptone and salt in the meat infusion broth by careful heating. Add indicator and glucose. Adjust to pH 7.0 to 7.5. Tube in fairly large tubes (20 by 1.5 cm.), the column of broth to be about 12 cm. deep. Add 3 pieces of calf

brain about 1 cm. square and 2 or 3 pieces of crushed marble to each tube (dip the pieces of brain in water before tubing to prevent sticking to the tubes). Autoclave at 121° C. for 20 minutes.

If the broth is to be used for blood cultures add 5 gms. of sodium citrate to 1000 cc. to prevent coagulation of the blood.

**Brown's Egg Cube Broth.**—For proteolytic study of anaerobes: From the white of a hard-boiled egg cut cubes of about 5 mm. Place a cube into each tube of infusion broth. Cover with a layer of sterile vaseline and autoclave at 121° C. for 15 minutes.

**Avery Blood Broth Medium.**—For the cultivation of pneumococci:

<i>Composition.</i> —Infusion broth (pH 7.6) . . . . .	90.0 cc.
Dextrose solution, 20 per cent. . . . .	5.0 cc.
Sterile rabbit blood. . . . .	5.0 cc.

*Preparation.*—Add the sterile dextrose and blood to the sterile broth. Incubate at 37° C. for 24 to 48 hours to test for sterility.

**Kracke Blood Culture Medium.**—For blood cultures:

<i>Composition.</i> —Heart muscle infusion. . . . .	750.0 cc.
Brain suspension . . . . .	250.0 cc.
Sodium citrate . . . . .	1.0 gm.
Dextrose . . . . .	10.0 gm.
Lacto-peptone . . . . .	10.0 gm.
Dibasic sodium phosphate. . . . .	2.0 gm.
Sodium chloride (C.P.). . . . .	4.0 gm.

*Preparation.*—Mix 500 gm. of finely ground beef heart muscle with 1000 cc. of distilled water. Allow to stand overnight in refrigerator. Press the infusion through a metal strainer. Heat without constant stirring. Pass the infusion through a small mesh wire gauze, or allow to settle and decant.

Macerate brain tissue and mix with water in same proportions as the heart muscle. Pass the infusion through a metal strainer. Heat the filtrate slowly to the boiling point, with constant stirring. Do not filter.

Mix all of the ingredients; heat to effect solution. Adjust to pH 7.4. Dispense 50 cc. in 100 cc. flasks. Autoclave at 121° C. for 20 minutes.

**Liver Infusion Broth.**—For the cultivation of *Brucellae*:

<i>Composition.</i> —Liver infusion . . . . .	500.0 cc.
Peptone . . . . .	10.0 gm.
Sodium chloride . . . . .	5.0 gm.
Water . . . . .	500.0 cc.

*Method of Preparation.*—Prepare the liver infusion by grinding 500 grams of fresh beef liver, free from fat, and immerse in 500 cc. of water. Heat in Arnold sterilizer for 30 minutes. Stir thoroughly. Heat for another 90 minutes. Filter through cheesecloth.

Heat the medium in the Arnold sterilizer until the solids are dissolved. Adjust the pH to 7.0. Heat in the Arnold sterilizer for 30 minutes. Decant off the clear fluid. Tube or put into flasks and autoclave at 121° C. for 30 minutes.

**Liver Infusion Medium (Cameron and Williams).**—For general cultivation of anaerobes. Requires petrolatum seal and does not cause much sporulation of anaerobes:



**Composition.**—Liver infusion (as described below) 1000.0 cc.

Peptone ..... 10.0 gm.

Dipotassium phosphate ..... 1.0 gm.

**Preparation.**—Steam 500 gm. of ground beef liver with 1000 cc. of tap water for 2 to 3 hours. Cool and strain through cheesecloth. Make filtrate to original volume and add 1 per cent peptone and 0.1 per cent dipotassium phosphate. Sterilize filtrate in flasks in autoclave for 30 minutes at 15 pounds pressure. Dry the tissue at 55 to 60° C. When the medium is needed, tube the infusion prepared above over small chunks of the dried tissue and resterilize in autoclave at 121° C. for 15 minutes.

**Corn-Liver Medium (McClung and McCoy).**—For cultivation and isolation of all types of anaerobic bacteria without the use of petrolatum seal. Particularly adapted for isolation and study of bacteria causing spoilage of canned food:

**Composition.**—Corn meal ..... 50.0 gm.

Dried liver ..... 20.0 gm.

Distilled water ..... 1000.0 cc.

**Components.**—Ordinary whole (yellow) corn meal is used. The dried liver is obtained by drying at 55 to 60° C. the liver tissue obtained from the preparation of Liver Infusion (Cameron and Williams) and grinding.

**Method of Preparation.**—Mix the corn meal, liver tissue and water, and steam 1 hour. This is cooled and tubed as desired. The tubed medium is sterilized in the autoclave at 121° C. for 2 hours. The pressure in the autoclave should be reduced slowly to prevent blowing of the plugs.

**Bile Brilliant Green Broth.**—For the detection of colon bacilli in milk and water:

**Composition.**—Beef bile ..... 200.0 cc.

Peptone ..... 10.0 gm.

Lactose ..... 10.0 gm.

Brilliant green (1 per cent aq. sol.) . 13.3 cc.

Distilled water to give ..... 1000.0 cc.

**Ingredients.**—The brilliant green dye should be of highest purity. The brand of peptone is probably of minor importance and any good peptone will do. Dried beef bile is to be preferred to fresh bile because it is easier to handle and more uniform.

**Method of Preparation.**—Dissolve the peptone and lactose in half the required distilled water. Add 20 gm. dehydrated ox bile or 200 cc. of fresh beef bile. Measure volume and titrate to pH 7.4. Add brilliant green (13.3 cc. of 1 per cent aq. solution per liter) and make up to 1000 cc. Distribute into Dunham tubes in 10 cc. amounts and autoclave at 121° C. for 20 minutes. If it is desired to test 10 cc. of water the concentration of ingredients must be increased to 1½ times that given. Such medium is tubed in 20 cc. amounts and 10 cc. of water added.

**Koser Citrate Medium.**—For differentiation between fecal and soil types of colon bacilli:

**Composition.**—Sodium ammonium phosphate (4H<sub>2</sub>O) ..... 1.5 gm.

Monobasic potassium phosphate (anhydrous) . . 1.0 gm.

Magnesium sulphate (anhydrous) ..... 0.2 gm.

Sodium citrate (2H<sub>2</sub>O) ..... 3.0 gm.

Distilled water ..... 1000.0 cc.

**Preparation.**—Simply dissolve ingredients, adjust pH to 6.7 to 6.9, tube, and sterilize at 121° C. for 30 minutes. No adjustment of reaction is necessary.

**Malonate Medium (Leifson).**—For the differentiation of the aerobacter and escherichia groups of colon bacilli:

<i>Composition.</i> —Ammonium sulphate .....	2.0 gm.
Dipotassium phosphate .....	0.6 gm.
Monopotassium phosphate .....	0.4 gm.
Sodium malonate .....	3.0 gm.
Bromthymol blue (0.2 per cent alc. sol.)	10.0 cc.
Distilled water .....	1000.0 cc.

**Preparation.**—Dissolve salts in distilled water and add 1.0 cc. of a 0.2 per cent alcoholic solution of bromthymol blue to each 100 cc. of medium. Adjust to pH 6.9 to 7.1. Tube and autoclave at 121° C. for 15 minutes.

**Methyl Red—Voges Proskauer Medium.**—For the differentiation of the aerobacter and escherichia groups of colon bacilli:

<i>Composition.</i> —Bacto-peptone .....	5.0 gm.
Dextrose .....	5.0 gm.
Dipotassium phosphate .....	5.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Dissolve the ingredients in the water by careful heating. Adjust to pH 6.8 to 7.1. Boil for 10 minutes. Adjust volume to 1000 cc. and filter through paper. Tube and autoclave at 121° C. for 20 minutes.

#### **Peptone Medium for Indol Production by Aerobic Bacteria.—**

<i>Composition.</i> —Bacto-tryptone .....	5.0 gm.
Sodium chloride .....	5.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Dissolve the bacto-tryptone and salt in the water by careful heating. Adjust to pH 6.5 to 7.5. If necessary filter through paper. Tube and autoclave at 121° C. for 15 minutes.

In conducting the indol test with hemophilic and other fastidious organisms aseptically add 2 cc. of sterile defibrinated blood to each 100 cc. of sterile medium. Tube aseptically and incubate at 37° C. for 48 to 72 hours for sterility.

#### **Peptone Medium for Indol Production by Clostridia (Reed and Orr).—**

<i>Composition.</i> —Bacto-tryptone .....	20.0 gm.
Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )....	2.0 gm.
Dextrose .....	1.0 gm.
Agar .....	1.0 gm.
Sodium thioglycollate .....	1.0 gm.
Water .....	1000.0 cc.

**Preparation.**—Dissolve the ingredients in the water by gentle heating. Filter through paper. Tube and autoclave at 121° C. for 15 minutes.

**Nitrate Medium for Nitrite Test with Aerobic Bacteria.—**

<i>Composition.</i> —Bacto-tryptone .....	10.0 gm.
Sodium chloride .....	5.0 gm.
Sodium nitrate .....	0.2 gm.
Distilled water .....	1000.0 cc.

*Preparation.*—Dissolve the ingredients in the water by gentle heating. Filter through paper. Adjust to pH 6.5 to 7.5. Autoclave at 121° C. for 15 minutes.

**Nitrate Medium for Nitrite Test with Clostridia (Reed and Orr).—**

<i>Composition.</i> —Bacto-tryptone .....	20.0 gm.
Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )....	2.0 gm.
Dextrose .....	1.0 gm.
Agar .....	1.0 gm.
Potassium nitrate .....	1.0 gm.
Distilled water .....	1000.0 cc.

*Preparation.*—Dissolve the ingredients in the water by gentle heating. Filter through paper. Tube and autoclave at 121° C. for 15 minutes.

**Sodium Hippurate Broth.—**For the test for hydrolysis of sodium hippurate:

<i>Composition.</i> —Infusion broth .....	1000.0 cc.
Sodium hippurate .....	10.0 gm.

*Preparation.*—Dissolve the sodium hippurate in the broth. Tube and mark the level of the medium with a “non-run” wax pencil or other means on each tube. Autoclave at 121° C. for 15 minutes.

**Lead Acetate Broth for Hydrogen Sulfide Production by Clostridia (Reed and Orr).—**

<i>Composition.</i> —Proteose peptone .....	20.0 gm.
Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )....	2.0 gm.
Dextrose .....	1.0 gm.
Agar .....	2.0 gm.
Water .....	1000.0 cc.

*Preparation.*—Dissolve, adjust to pH 7.6 and add 10 cc. of 2 per cent lead acetate. Tube and autoclave at 121° C. for 15 minutes. This results in a cloudy precipitate which, however, remains after autoclaving in reasonably stable suspension.

**Milk with Indicator.**—*Preparation.*—To 1000 cc. of skimmed milk add 5 cc. of 0.2 per cent solution of bromcresol purple. Or add a sufficient amount of litmus solution to produce a distinct blue color. Dispense in tubes and sterilize in Arnold for 20 minutes on each of 3 days in succession.

**Blood Milk.**—For the cultivation of hemophilic organisms:

*Preparation.*—Add 20 cc. of sterile blood to 100 cc. of sterile 0.85 per cent saline solution and heat just to boiling. Add to 1000 cc. of sterile skimmed milk (sterilized in Arnold for 20 minutes on each of 3 days in succession). Add 5 cc. of 0.2 per cent solution of bromcresol purple. Tube aseptically and incubate 48 to 72 hours to test sterility

**Iron Milk (Reed and Orr).**—For the identification of anaerobic bacilli:

*Preparation.*—Mix 100 gms. Bacto dried milk with 1000 cc. of cold water. Strain through gauze. Adjust to pH 6.8. Place 10 cc. in tubes to which 0.1 gm. of reduced iron (Merck's "reduced with hydrogen") had previously been added. Autoclave at 121° C. for 30 minutes.

**Leifson Selenite-F Enrichment Medium.**—For the isolation of typhoid and paratyphoid bacilli from feces and urine:

<i>Composition.</i> —Sodium hydrogen selenite (anhydrous) . . . . .	4.0 gm.
Peptone . . . . .	5.0 gm.
Lactose . . . . .	4.0 gm.
Sodium phosphate (anhydrous) . . . . .	10.0 gm.
Distilled water . . . . .	1000.0 cc.

*Preparation.*—Mix and warm to dissolve the ingredients. Determine experimentally the exact proportions of monosodium phosphate and disodium phosphate which, together with the particular kind of peptone used and a particular lot or make of sodium selenite, will give a reaction of pH 7.0 to 7.1. Dispense in tubes and sterilize in Arnold for 20 minutes on each of 3 days in succession.

**Beerwort Medium.**—For the cultivation of yeasts: Obtain hopped beerwort at a brewery and autoclave at 121° C. for 15 minutes. Cool, filter and tube. Carbohydrates may also be added to make 2 per cent solutions.

**Sabouraud's Broth (Modified).**—For the cultivation of yeasts and fungi:

<i>Composition.</i> —Peptone . . . . .	10.0 gm.
Dextrose (for fungi) or maltose (for yeasts) . . . . .	40.0 gm.
Sodium chloride . . . . .	7.5 gm.
Beef extract . . . . .	3.5 gm.
Water . . . . .	1000.0 cc.

*Preparation.*—Dissolve the solids with slow heating and filter. Adjust to pH 5.6. Tube and autoclave at 121° C. for 15 minutes.

**Gelatin Medium for Aerobic Organisms.**—For purposes of cultivation and the determination of liquefaction:

<i>Composition.</i> —Distilled water . . . . .	1000.0 cc.
Gelatin . . . . .	120.0 gm.
Beef extract . . . . .	3.0 gm.
Bacto-peptone . . . . .	5.0 gm.

*Preparation.*—Combine and heat slowly to 65° C. in a water-jacketed container until the ingredients are dissolved. Make up to 1000 cc. with water. Adjust to pH 7.0. Heat to boiling while stirring vigorously. Make up to 1000 cc. and filter while hot through paper pulp. Dispense in tubes and autoclave at 121° C. for 15 minutes.

**Gelatin Medium for Clostridia (Reed and Orr).**—

<i>Composition.</i> —Gelatin . . . . .	50.0 gm.
Bacto-peptone . . . . .	10.0 gm.
Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) . . . . .	2.0 gm.
Dextrose . . . . .	1.0 gm.
Sodium thioglycollate . . . . .	1.0 gm.
Water . . . . .	1000.0 cc.

**Preparation.**—Dissolve the ingredients in the water with gentle heating. Filter through paper. Tube and autoclave at 121° C. for 20 minutes.

**Semisolid Agar.**—For the cultivation of meningococci:

<i>Composition.</i> —Agar .....	1.5 gm.
Dextrose .....	1.0 gm.
Potassium chloride .....	0.2 gm.
Calcium chloride .....	0.1 gm.
Beef infusion broth .....	1000.0 cc.

**Preparation.**—Add the agar and dextrose to the broth and heat to 100° C. Add the potassium chloride and calcium chloride; continue heating; stir or agitate until dissolved. Adjust to pH 7.3. Dispense in tubes and autoclave at 121° C. for 15 minutes. Incubate at 37° C. for 24 to 36 hours as test for sterility.

**Carbohydrate Semisolid Agar for Fermentation Tests.**—For fermentation studies with meningococci, gonococci or other Neisseriae:

<i>Composition.</i> —Agar .....	1.5 gm.
Beef infusion broth .....	1000.0 cc.

**Preparation.**—Add the agar to the broth and dissolve by boiling. Adjust volume to 1000 cc. and the pH to 7.6 to 7.8. Add 5 cc. of 0.2 per cent alcoholic solution of bromthymol blue. The color should correspond to pH 7.6 in the bromthymol blue color range. Tube in 5 cc. amounts and autoclave at 121° C. for 15 minutes.

Before use, melt the agar in the tubes by placing them in boiling water and add aseptically 0.5 cc. of a sterile 5 per cent solution of the desired sugar which gives a concentration of 0.5 per cent. The sugar solution may be sterilized by filtration or by Arnold sterilizations for 20 minutes on each of 3 days in succession.

**Extract Agar.**—For the general cultivation of the less fastidious organisms:

<i>Composition.</i> —Beef extract .....	3.0 gm.
Bacto-peptone .....	10.0 gm.
Agar .....	20.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Mix thoroughly, and boil to dissolve the ingredients. Adjust to pH 7.2 to 7.4. Boil again and filter through absorbent cotton into tubes. Autoclave at 121° C. for 20 minutes.

**Extract Agar (A.P.H.A. Standard Method).**—For the enumeration of bacteria in milk and water:

<i>Composition.</i> —Beef extract .....	3.0 gm.
Bacto-peptone .....	5.0 gm.
Agar .....	15.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Mix the ingredients and boil until all of the agar is dissolved. Make up to 1000 cc. with hot distilled water. Adjust pH to about 7.0. Heat to boiling and filter if necessary. Distribute in tubes or flasks and autoclave at 121° C. for 20 minutes.

**Beef Infusion Agar.**—For the general cultivation of the less fastidious bacteria:

<i>Composition.</i> —Agar .....	20.0 gm.
Bacto-peptone .....	10.0 gm.
Sodium chloride (C.P.) .....	5.0 gm.
Agar .....	20.0 gm.
Beef infusion broth .....	1000.0 cc.

*Preparation.*—Dissolve the ingredients in the infusion broth by heating. Adjust to pH 7.4 to 7.6. Filter through non-absorbable cotton. Dispense and autoclave at 121° C. for 20 to 30 minutes.

**Blood Agar.**—For the cultivation of fastidious organisms and the differentiation of streptococci:

*Preparation.*—Melt beef or heart infusion agar which has a reaction of pH 7.4 to 7.6 and cool to 45° C. To each 450 cc. add 50 cc. of sterile defibrinated human, horse, or rabbit blood. Mix and transfer aseptically to sterile Petri dishes or tubes (all to harden as slants). Incubate at 37° C. for 24 to 36 hours as a test for sterility.

*Dextrose blood agar* may be prepared in the same manner by adding 25 cc. of a 20 per cent sterile solution of dextrose to 500 cc. of the infusion-blood agar before it has hardened (approximately 1 per cent dextrose). The dextrose solution may be prepared by dissolving 20 gms. of dextrose in 100 cc. of distilled water and sterilizing by filtration or in Arnold sterilizer for 20 minutes on each of 3 days in succession.

**“Chocolate” Blood Agar.**—For the cultivation of the fastidious bacteria:

*Preparation.*—This medium is prepared in the same manner as blood agar with or without dextrose. Transfer aseptically to sterile tubes or Petri dishes. Place in Arnold sterilizer and allow the temperature to rise very slowly until the medium has a definite chocolate color. Excessive heating will cause the blood to coagulate and the finished medium will show clumps of cooked blood instead of the uniform, smooth, chocolate color desired. Allow to cool and incubate at 37° C. for 24 to 36 hours to check sterility before use.

**Proteose “Chocolate” Agar.**—For the cultivation of gonococci and other fastidious bacteria:

*Preparation.*—1. Dissolve 9 gm. Bacto-proteose No. 3 agar in 100 cc. of water with slow heating. Mix well and autoclave at 121° C. for 20 minutes.

2. Dissolve 2 gm. Bacto-hemoglobin in 100 cc. of water heated to 50° C. When solution is nearly complete, filter through cheesecloth. Autoclave at 121° C. for 15 minutes.

3. Cool both solutions to 50° to 60° C. and mix aseptically in equal volumes. Pour into sterile Petri dishes. Cool. Incubate at 37° C. for 24 to 36 hours to test for sterility.

**Avery Sodium Oleate Blood Agar.**—For the isolation of *Hemophilus influenza*:

*Preparation.*—Prepare a 2 per cent solution of sodium oleate in distilled water and autoclave at 121° C. for 30 minutes.

Prepare a suspension of erythrocytes by centrifuging aseptically sterile defibrinated human or rabbit blood, removing the serum and adding an equal volume of sterile broth.

Melt sterile infusion agar (pH 7.4) in boiling water, cool to 45° C. and to each 94 cc. add 5 cc. of the sterile sodium oleate solution and 1 cc. of the blood cell sus-

pension. Dispense aseptically in sterile Petri dishes. Incubate at 37° C. for 24 hours as a test for sterility.

**Bordet-Gengou Potato Blood Agar (Modified).**—For the cultivation of *Haemophilus pertussis*:

**Preparation.**—Boil 500 gms. of peeled, sliced potatoes in 1000 cc. of distilled water plus 40 cc. of glycerin until soft. Make up volume with water, strain through gauze and allow to stand for sedimentation. Decant off the supernatant fluid.

To each 500 cc. of the clear potato extract add 1500 cc. of a 0.75 per cent solution of C.P. sodium chloride in water and 50 gms. of agar. Allow the mixture to stand for 15 minutes. Heat until the agar is dissolved and dispense. It is not necessary to adjust the reaction which is usually somewhat less than pH 6.5. Autoclave at 121° C. for 25 minutes. This base may be stored for several months.

For use, melt the base in boiling water and cool to 45° C. To each 100 cc. add 20 cc. of sterile defibrinated blood (never over 72 hours old). Mix by whirling and dispense aseptically in sterile Petri dishes or tubes (allow to harden as slants). Incubate at 37° C. for 24 to 36 hours as a test for sterility. Satisfactory plates should be bright cherry red in color and free from bubbles and lumps of agar. Excessive heating of the basic medium should be avoided. Best results are obtained by adding the blood to the melted agar after the first sterilization.

**Francis Blood-Glucose-Cystine Agar.**—For the cultivation of *Bact. tularensis* and *Brucella*:

**Preparation.**—Melt 500 cc. of beef infusion agar (pH 7.4). Add 5 gms. glucose and 0.5 gm. cystine. Autoclave at 121° C. for 20 minutes. Cool to 50° C. and add 25 cc. of sterile defibrinated blood (rabbit or human). Heat in a water bath at 60° C. for 2 hours. Dispense aseptically in sterile Petri dishes or tubes (allow to harden as slants). Incubate at 37° C. for 24 to 36 hours as a test for sterility.



FIG. 149.—SERUM INSPISSATOR

**Serum or Ascitic Carbohydrate Agar.**—

The carbohydrates commonly used are dextrose, maltose, sucrose, and lactose.

**Preparation.**—Melt infusion agar (pH 7.4) and cool to 50° C. To each 100 cc. add 5 cc. of a sterile 20 per cent solution of the carbohydrate in distilled water (sterilized by filtration or in Arnold for 20 minutes on each of 3 days in succession), 5 cc. of sterile serum (horse or human)

or 10 cc. of sterile, bile-free ascitic fluid and 1 cc. of sterile solution of Andrade's indicator. Mix by whirling and dispense aseptically in sterile test tubes. Replace cotton plugs by sterile rubber stoppers and allow to harden in slanting position. Incubate at 37° C. for 24 to 36 hours to test sterility.

**Löffler Blood Serum Medium.**—For the isolation and cultivation of diphtheria bacilli and for the study of pigment production and proteolysis:

1. Dissolve 2.5 gms. of dextrose in 250 cc. of beef infusion broth (pH 7.6). Add 750 cc. of clear horse, bovine, sheep or hog serum. Adjust to pH 8.3.

2. Place 4 to 5 cc. in test tubes. Place in autoclave in a slanting position. Close the door and fasten tightly. Close the air valve and autoclave at 121° C. for 10 minutes,

letting no air or steam escape. Now open the outlet valve to let air escape and simultaneously let in steam. When all the air has escaped close the valve and sterilize at 121° C. for 1½ hours. The final pH will be 7.6 to 7.8. The medium may also be coagulated at 80 to 90° C. in an inspissator (Fig. 149) or Arnold, after which it is sterilized by heating at 100° C. for 20 minutes on 3 successive days.

**Douglas Potassium Tellurite Medium (Gilbert Modification).**—For the rapid identification of diphtheria bacilli:

1. Dissolve 15 gms. of granulated agar in 1000 cc. of beef infusion broth. Adjust to pH 7.2 to 7.4.

2. Place in bottles or flasks without filtration and sterilize in autoclave at 121° C. for 20 minutes.

3. Prepare a 10 per cent solution of dextrose in water and sterilize in Arnold for 1 hour.

4. Prepare a 1 per cent solution of potassium tellurite in water and sterilize in Arnold for 1 hour.

5. When needed, melt the agar base in an Arnold sterilizer and cool to 50° C. To each 100 cc. add 5 cc. of sterile human or hog serum, 2 cc. of sterile dextrose solution and 1 cc. of sterile potassium tellurite solution. Mix and distribute in sterile Petri dishes. Incubate at 37° C. for 24 to 36 hours for sterility.

6. *Tellurite "chocolate" agar* may be prepared in the same manner by substituting 10 cc. of sterile defibrinated human or rabbit blood in place of serum per 100 cc. of agar base.

**Testicular Hydrocele Agar (Pitts).**—For the isolation and cultivation of gonococci and meningococci:

1. Infuse 500 gms. of minced sheep or beef testicles in 1000 cc. of distilled water overnight in the refrigerator. Strain through gauze, boil vigorously for 30 minutes and strain through gauze. Restore volume with distilled water and boil for 30 minutes. Strain through gauze and filter through paper. Restore volume and add:

Bacto-peptone .....	20.0 gm.
Sodium chloride .....	5.0 gm.
Dextrose .....	5.0 gm.

2. Adjust to pH 7.8 (pH should be between 7.4 and 7.6 after autoclaving) and add 20 gm. of bacto-agar.

3. Autoclave at 121° C. for 20 minutes, filter through gauze and cotton and tube approximately 5 cc. per tube.

4. Autoclave at 121° C. for 30 minutes, cool to 50° C. and add 2 cc. of sterile hydrocele fluid to each tube. Rotate to insure even mixture and slant. Or to 10 cc. add 4 cc. of sterile hydrocele fluid, mix and pour into sterile Petri dishes.

5. After the agar has hardened, replace the cotton stoppers with rubber ones.

**Bacto-Tryptose Agar.**—For the cultivation of gonococci and other fastidious bacteria:

<i>Composition.</i> —Bacto-tryptose .....	20.0 gm.
Dextrose .....	1.0 gm.
Sodium chloride .....	5.0 gm.
Agar .....	20.0 gm.
Water .....	1000.0 cc.



**Preparation.**—Dissolve the solids with slow heating. Adjust the pH to 7.0. Boil for 1 minute, filter, tube or place in flasks and autoclave at 121° C. for 20 minutes.

**Cohn Cysteine Monohydrochloride Serum Hemoglobin Agar.**—For the cultivation of gonococci:

1. Dissolve 55 gms. of Difco proteose No. 3 agar and 2.0 gm. of cysteine monohydrochloride in 1000 cc. of hot distilled water. Filter through gauze or cotton.
2. Autoclave at 121° C. for 20 minutes.
3. Cool to 50° C. and add 160 cc. of a sterile 5 per cent solution of hemolyzed blood (horse, beef, sheep or human in distilled water) and 70 cc. of sterile serum.
4. Dispense in sterile Petri dishes carrying 25 cc. of the medium. Incubate at 37° C. for 24 to 36 hours for sterility. Keep in refrigerator.

**B-B-L Anaerobic Agar.**—To be used with the Brewer Petri dish cover method for the cultivation of anaerobes:

<i>Composition.</i> —Polypeptone BBL .....	20.0 gm.
Sodium chloride .....	5.0 gm.
Dextrose .....	10.0 gm.
Agar (powdered) .....	20.0 gm.
Sodium thioglycollate .....	2.0 gm.
Sodium formaldehyde sulfoxylate .....	1.0 gm.
Methylene blue .....	0.002 gm.

**Preparation.**—Suspend 58.0 gms. of the powder in 1000 cc. of distilled water. Allow to soak 5 to 10 minutes. Heat gently until the mixture boils and the powder is dissolved. Dispense in tubes and autoclave at 121° C. for 20 minutes.

**Eosin Methylene Blue Agar.**—For isolation of intestinal pathogens:

<i>Composition.</i> —Peptone .....	10.0 gm.
Dipotassium phosphate .....	2.0 gm.
Lactose .....	5.0 gm.
Sucrose .....	5.0 gm.
Eosin y .....	0.2 gm.
Methylene blue .....	0.05 gm.
Agar .....	15.0 gm.
Water .....	1000.0 cc.

**Preparation.**—Dissolve the peptone and phosphate in distilled water by careful heating. Titrate to pH 7.2, boil a few minutes and filter, if necessary. Add the agar, allow to soak a few minutes and boil until dissolved. Adjust volume. Add the lactose and sucrose. To each 100 cc. of medium add 1 cc. of a 2 per cent solution of eosin y and 1 cc. of a 0.5 per cent solution of methylene blue. Tube in 15 cc. amounts or put into flasks and sterilize in the autoclave at 121° C. for 20 to 30 minutes.

**Desoxycholate Agar.**—For isolation of intestinal pathogens:

<i>Composition.</i> —Peptone .....	10.0 gm.
Agar .....	16.0 gm.
Sodium desoxycholate .....	1.0 gm.
Sodium chloride .....	5.0 gm.
Dipotassium phosphate .....	2.0 gm.

Lactose .....	10.0 gm.
Ferric ammonium citrate .....	2.0 gm.
Neutral red (certified) 0.1 per cent alc. sol. . .	33.3 cc.
Distilled water .....	1000.0 cc.

**Preparation.**—Dissolve the peptone in distilled water by gentle heating. Titrate to pH 7.5, boil and filter, if necessary. Dissolve agar (use powdered agar) in the peptone solution and dissolve by boiling. Add the other ingredients in the order given and dissolve. Adjust volume and titrate to pH 7.5. Add neutral red (33.3 cc. of 0.1 per cent alc. solution per 1000 cc. of medium). Distribute in tubes or flasks and Arnoldize for 15 minutes. Spore formers will not grow in this medium and the sterilization need only kill vegetative cells.

**Leifson Desoxycholate Citrate Agar.**—For isolation of intestinal pathogens:

**Preparation.**—Add 1200 cc. of distilled water to 400 gms. of fresh, lean ground pork. Infuse at room temperature for 1 hour. Add 2 cc. of normal hydrochloric acid with stirring and boil thoroughly for 5 minutes.

Strain through cheesecloth and filter through paper until clear and free from visible fat. Add normal sodium hydroxide solution to pH 8.0, boil again for 10 minutes, and filter through paper. Add water to make 1200 cc., adjust to pH 7.4 and autoclave at 121° C. for 30 minutes. Store in refrigerator.

As required, add 1 gm. peptone and 2 gms. agar to 100 cc. Boil for 3 minutes, filter, and restore to 100 cc. Allow to stand 15 minutes and add 1 gm. lactose, 2 gms. sodium citrate, 0.2 gms. ferric ammonium citrate and 0.5 gm. sodium desoxycholate. Adjust to pH 7.3 to 7.5. Add 2.0 cc. of 0.1 per cent aqueous solution of neutral red. Sterilize in Arnold for 15 minutes and dispense in sterile Petri dishes (preferably with porous top), about 15 cc. to each. Heat is detrimental to the medium and the less it is heated the better. Store the plates in a refrigerator in the dark. It is advisable to use the plates within 4 days after preparation.

**Desoxycholate Lactose Agar.**—For direct enumeration of colon bacilli in milk and water:

Peptone .....	10.0 gm.
Agar .....	15.0 gm.
Lactose .....	10.0 gm.
Sodium desoxycholate .....	0.5 gm.
Sodium chloride .....	5.0 gm.
Sodium citrate .....	2.0 gm.
Neutral red (certified) 0.1 per cent sol. . .	35.0 cc.
Distilled water .....	1000.0 cc.

Use a good general peptone such as Wilson CB, Proteose, Fairchild, etc.

Dissolve peptone in distilled water by careful heating and titrate to pH 7.3 to 7.5. Boil and filter, if necessary. Add powdered agar and dissolve by boiling. Dissolve other ingredients in the melted agar, adjust volume and check pH (7.3 to 7.5). Add neutral red (35 cc. of a 0.1 per cent solution). Dispense in tubes or flasks as desired and autoclave at 121° C. for 15 to 30 minutes depending upon volume.

**Endo Agar (Robinson and Bettger).**—For the isolation of intestinal pathogens:

**Preparation.**—To 1000 cc. of beef extract agar add 10 cc. of 10 per cent aqueous solution of sodium carbonate. Adjust to pH 7.6 to 8.0 and steam in an Arnold for

10 minutes. Add 10 gm. lactose, 10 cc. of 10 per cent aqueous solution of sodium bisulfite and 0.5 to 3 cc. of 10 per cent alcoholic solution of basic fuchsin. Sterilize by steam at 115° to 116° C. for 20 minutes. Store in refrigerator in the dark.

As required melt and distribute 15 to 20 cc. amounts in sterile Petri dishes with porous tops. Use within 4 or 5 days.

**Russell's Double Sugar Agar.**—For differentiation of intestinal bacilli:

Peptone .....	10.0 gm.
Beef extract .....	3.0 gm.
Lactose .....	10.0 gm.
Dextrose .....	1.0 gm.
Sodium chloride .....	5.0 gm.
Agar .....	15.0 gm.
Phenol red (0.2 per cent sol.) .....	12.5 cc.
Distilled water .....	1000.0 cc.

To melted extract agar add the lactose and dextrose. Titrate to pH 7.6 and add 12.5 cc. of a 0.2 per cent solution of phenol red to each 100 cc. of medium.

**Phenol Red Tartrate Agar.**—For differentiation among the paratyphoid bacilli:

Peptone .....	10.0 gm.
Sodium potassium tartrate .....	10.0 gm.
Sodium chloride .....	5.0 gm.
Agar .....	15.0 gm.
Phenol red (0.2 per cent sol.) .....	12.5 cc.
Distilled water .....	1000.0 cc.

Adjust to pH 7.6. Dissolve the salts and peptone in distilled water by careful heating. Titrate to pH 7.8. Add powdered agar and allow to soak for a few minutes. Boil for 2 to 3 minutes to dissolve agar. Add 12.5 cc. of a 0.2 per cent solution of phenol red. Adjust volume, tube and autoclave at 121° C. for a period of 15 minutes.

**Lead Acetate Agar.**—For study of hydrogen sulfide production:

<i>Composition.</i> —Peptone .....	20.0 gm.
Beef extract .....	3.0 gm.
Dextrose .....	1.0 gm.
Basic lead acetate .....	0.5 gm.
Agar .....	15.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Dissolve the peptone in distilled water by careful heating. Titrate to pH 7.2, boil a few minutes, and filter through paper, if necessary. To the peptone solutions add the dextrose and powdered agar. Allow agar to soak a few minutes and dissolve by boiling for 2 to 3 minutes. Adjust volume and add 10 cc. of a 0.5 per cent aq. solution of basic lead acetate to each 100 cc. of the agar. Tube and sterilize by autoclaving at 121° C. for 15 minutes.

**King and Lucas Medium.**—For the cultivation of tubercle bacilli:

Use canned lima beans. Remove from can and drain off the juice. Mix 30 cc. of the bean juice with 180 cc. of distilled water. Press the beans through a sieve (wire gauze is satisfactory). Add 105 gms. of the mashed beans to the diluted juice and

autoclave for 20 minutes at 15 lbs., 120° C. Add 7 cc. glycerine immediately and cool to about room temperature. Add an equal volume of well mixed whole eggs and mix thoroughly. The eggs should be sterilized in 70 per cent alcohol for 10 minutes and broken aseptically into a sterile beaker. Add 1 cc. of 1 per cent alcoholic solution of gentian violet to each 100 cc. of medium (final concentration 1:10,000). Dispense into sterile test tubes with a sterile pipet and coagulate and sterilize by heating at 70° C. for 1 hour on 3 successive days. Incubate to check the sterility.

**Kligler Iron Agar.**—For the detection of hydrogen sulfide; also, whether or not the microorganisms attack lactose and whether acid or acid and gas are produced when the organisms attack glucose. However, with those organisms which readily produce  $H_2S$  the intense black color masks any change in the color of the indicator produced by the fermentation of the lactose or glucose and also the bubbles of gas in the medium if acid and gas are produced during the break down of the carbohydrate. It is best to use this medium only for the detection of  $H_2S$  and use a separate tube of Russell's medium for determining the reactions of the organisms towards lactose and glucose.

The Kligler medium supplied by the Difco Co. in the dehydrated form gives very good results. It contains in each 1000 cc. of medium bacto-tryptone, 20 gms.; lactose, 10 gms.; glucose, 1 gm.; sodium chloride, 5 gms.; ferric ammonium citrate, 0.5 gms.; sodium thiosulfate, 0.5 gms.; phenol red, 0.025 gms. and agar, 15 gms.

**Whey Agar.**—For the cultivation of lacto bacilli:

<i>Composition.</i> —Bacto-peptone .....	5.0 gm.
Agar .....	15.0 gm.
Skimmed milk .....	1000.0 cc.

*Preparation.*—Autoclave the skimmed milk at 121° C. for 3 hours. Filter off the coagulated protein and add the peptone and agar to the filtrate. Allow to soak for a few minutes and heat carefully to boiling. Boil to dissolve the agar, restore volume to 1000 cc. with distilled water and adjust to pH 6.0 to 6.5. Tube and autoclave at 121° C. for 20 minutes.

**Tomato Juice Agar.**—For the cultivation of *B. acidophilus*:

<i>Composition.</i> —Tomato juice (from canned tomatoes) .....	400.0 cc.
Peptonized milk (Difco) .....	10.0 gm.
Peptone .....	5.0 gm.
Agar .....	11.0 gm.
Distilled water .....	600.0 cc.

*Preparation.*—The tomato juice is obtained from commercial canned tomatoes. Pour the contents of the can into a large funnel with a coarse filter paper. Use the clear yellow juice filtrate.

Heat the mixture of tomato juice, peptonized milk and peptone to dissolve the ingredients.

Adjust the reaction of this mixture to pH 6.8 before adding the agar to it. Filter through paper. The final pH after sterilization will be about 6.6.

Add the agar to the water and boil or autoclave to dissolve the agar.

Combine these two mixtures while hot.

Filter through cotton.

Dispense in tubes or flasks and sterilize in the autoclave at 121° C. for 8 minutes. Remove the medium from the autoclave as soon as possible.

**Liver Infusion Agar.**—For cultivation and isolation of *Brucella*:

Liver infusion .....	500.0 cc.
Peptone .....	10.0 gm.
Sodium chloride .....	5.0 gm.
Agar .....	20.0 gm.
Distilled water .....	500.0 cc.

A. Liver Infusion—Fresh beef liver, free from fat, is ground and mixed with 500 cc. of water (Huddleston specifies tap water, but such water being variable it may be better to use distilled water). The mixture is heated in flowing steam for 20 minutes when it is thoroughly stirred. The heating is then continued in flowing steam for 1½ hours. Filter through wire screen.

B. Add the solid ingredients to the infusion and water and heat in Arnold for 1 hour. Remove and cool to 60° C. and adjust pH to 7.0. Heat again in Arnold for ½ hour. Decant off the clear agar. Put into tubes or flasks and sterilize at 121° C. for 30 minutes. The final pH will be about 6.6.

**Liver Infusion Agar with Thionin or Basic Fuchsin.**—For the differentiation of *Brucella*:

1. Prepare liver infusion agar as described above and adjust the pH to exactly 6.6.

2. Prepare stock solutions of 1 per cent thionin and 0.1 per cent basic fuchsin in water. Sterilize the dye solutions in the Arnold sterilizer for 20 minutes. Shake well and add to the melted agar.

3. Add 2.5 to 5.0 cc. of thionin per liter of agar, and 10 cc. of basic fuchsin per liter. Pour and cool. Incubate at 37° C. until the water of condensation evaporates.

**Potato Medium.**—For differentiation of bacteria.

*Preparation.*—1. Wash and peel large potatoes. Cut out cylindrical pieces with a cork-borer.

2. Then cut the cylinders diagonally with a knife so as to make slants. Immediately immerse the slants in distilled water. Change the water several times and soak the slants overnight in the refrigerator. Wash the slants again in fresh distilled water. Place the slants into test tubes and sterilize in the autoclave.

(The washing and soaking of the potato in water serves to prevent darkening of the potato. A small pledget of wet absorbent cotton in the end of each tube below the potato slant will help to prevent drying during storage.)

**Dorset Egg Medium (Modified).**—For cultivation of tubercle bacilli.

*Preparation.*—1. Soak 6 fresh eggs in 1:1000 mercuric chloride solution and allow the antiseptic to drain off on a sterile towel.

2. Break the eggs carefully and, as nearly aseptically as possible, add the yolks and whites to 100 cc. of sterile 5 per cent glycerol in distilled water contained in a wide mouth flask or beaker. Break up the yolks and mix thoroughly by means of a sterile spatula and by shaking the flask but avoid foaming. Tube, slant and inspissate for about 2 hours at 70 to 75° C. on 3 successive days or slant in a horizontal autoclave, close the autoclave tight and, without allowing the air to escape, autoclave at 121° C. for 15 minutes on each of 3 successive days. The original Dorset medium

did not contain glycerol, but there are many modifications of the medium. The one described resembles that of Soparkar (*Ind. J. Med. Res.*, 1916-17, iv, 28) except that it contains less egg.

**Petragnini Medium.**—For isolation of tubercle bacilli:

<i>Preparation.</i> —Potato (peeled and cut into small pieces).....	75.0 gm.
Milk (cream removed) .....	150.0 cc.
Potato flour .....	6.0 gm.
Peptone .....	10.0 gm.

Mix and heat in a double boiler for 10 minutes with frequent stirring. After mixture becomes pasty, continue to heat for 1 hour. Add sterile distilled water to make up volume. Cool to 50° C. To above add the following mixture:

Eggs (whole) .....	4
Egg yolk .....	1
Glycerol .....	12 cc.
Malachite green (2.0 per cent aqueous).....	10 cc.

Mix thoroughly and filter through sterile gauze into a sterile distributing funnel. Distribute into rather large test tubes. Place in a slanted position in an inspissator or Arnold and heat for 2 hours at from 70 to 75° C. on 3 successive days.

**Petroff Medium.**—For the cultivation of tubercle bacilli:

*Preparation.*—1. To 425 cc. of water add 500 gm. chopped beef or veal and 75 cc. of glycerol. Mix well. Keep in a cool place for 24 hours. Filter through gauze and collect the fluid in a sterile beaker.

2. Sterilize the shells of 6 eggs by placing them in 70 per cent alcohol for 15 minutes. Break the eggs into a sterile beaker, mix well and filter through gauze.

3. Mix 66 cc. of egg with 33 cc. of glycerol beef extract and add 1 cc. of a 1 per cent alcoholic solution of gentian violet. Tube and inspissate at 85° C. Heat for 1 hour at 75° C. on 3 successive days. Incubate at 37° C. for 24 to 48 hours as a test for sterility.

**Corper and Uyei's Potato Medium.**—For the cultivation of tubercle bacilli:

*Preparation.*—1. Select large white potatoes and scrub thoroughly. Cut cylinders about  $\frac{5}{8}$  inch in diameter with an apple corer. Remove the skin from the ends of the cylinders, and by an oblique cut, make wedge-shaped pieces about  $1\frac{1}{2}$  inches long. Keep under water while preparing to prevent discoloration.

2. Soak for 2 hours in a 1 per cent solution of sodium carbonate containing 1/75,000 part of crystal violet. (The dye and sodium carbonate should be mixed just prior to use. About 160 pieces of potato may be used in 1 liter of carbonate solution.) Wipe the potatoes with a clean towel and place in test tubes. Add 1.5 cc. of 6 per cent glycerol broth.

3. Plug and autoclave at 121° C. for 30 minutes. Incubate at 37° C. for 48 hours to test for sterility.

**Long-Seibert Synthetic Medium.**—For the cultivation of tubercle bacilli:

<i>Composition.</i> —Asparagin .....	5.0 gm.
Ammonium citrate .....	5.0 gm.
Potassium acid phosphate .....	3.0 gm.

Sodium carbonate (anhydrous) .....	3.0 gm.
Sodium chloride .....	2.0 gm.
Magnesium sulphate .....	1.0 gm.
Ferric ammonium citrate .....	0.05 gm.
Glycerol .....	50.0 cc.
Water .....	1000.0 cc.

**Preparation.**—Dissolve the ingredients in the water with slow heating. Filter, tube or place in a flask and autoclave at 121° C. for 15 minutes.

**Sabouraud's Maltose Agar (Fisher and Arnold).**—For the cultivation and identification of fungi:

Peptone .....	10.0 gm.
Maltose (technical) .....	40.0 gm.
Sodium chloride .....	7.5 gm.
Beef extract (Difco) .....	3.5 gm.
Agar .....	20.0 gm.
Distilled water .....	1000.0 cc.

Adjust to pH 5.4.

**Sabouraud's Dextrose Agar (Modified by Weidman).**—For routine use in the isolation of fungi:

<b>Composition.</b> —Peptone (Fairchild) .....	10.0 gm.
Dextrose (crude American) .....	40.0 gm.
Agar .....	18.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Add the agar to 700 cc. of water and soak for an hour. Add the peptone and dextrose to 300 cc. of water and dissolve. Cook the agar and water in an aluminum pot, stirring occasionally. When the agar is almost dissolved, start to cook the peptone and dextrose mixture; stir. When both the agar and the peptone and dextrose are dissolved, add the peptone and dextrose to the agar and stir. The pH is usually 5.6 although it is not necessary to titrate. Dispense and autoclave at 121° C. for 20 minutes.

**Conservation Agar** for preserving cultures of fungi is prepared in the same manner except that dextrose is omitted.

**Corn meal Agar.**—For differentiating various species of *Monilia* and *Cryptococcus*. It is also useful in studying spore forms of the dematophytes:

<b>Composition.</b> —Yellow corn meal .....	40.0 gm.
Agar .....	15.0 gm.
Distilled water .....	1000.0 cc.

**Preparation.**—Add the corn meal to 500 cc. of water and keep heated to 65° C. for 1 hour. Filter through paper. Dissolve the agar in 500 cc. of water by heating. Mix the corn meal and the agar. Filter through cotton while the agar is still hot. Dispense and autoclave at 121° C. for 20 minutes.

**Potato-Carrot Agar.**—Useful for demonstrating the color of colonies of fungi:

<i>Composition.</i> —Carrots .....	20.0 gm.
Potatoes .....	20.0 gm.
Agar .....	15.0 gm.
Distilled water .....	1000.0 cc.

*Preparation.*—Wash and peel the vegetables and cut them into small pieces; add them to 700 cc. of water and boil down the mixture to 500 cc. Filter through paper. Dissolve the agar in 500 cc. of water by heating. Mix the vegetables and the agar. Dispense and autoclave at 121° C. for 20 minutes.

**Honey Agar.**—For the cultivation and identification of fungi:

Honey .....	60.0 gm.
Peptone .....	10.0 gm.
Agar .....	20.0 gm.
Distilled water .....	1000.0 cc.

Adjust to pH 5.5.

Sterilize by the fractional method.

**Beerwort Agar.**—For the cultivation of yeasts:

Dissolve 7.5 gm. of agar in the beerwort medium with slow heating. Filter, tube and autoclave at 121° C. for 20 minutes.

**Triple N.N.N. Medium.**—For the cultivation of the Leishmania (Novy, MacNeal and Nicolle):

Agar .....	14.0 gm.
Sodium chloride .....	6.0 gm.
Distilled water .....	900.0 cc.

After tubing and sterilizing in the autoclave, the medium is cooled to about 50° C., and  $\frac{1}{3}$  volume of sterile whole rabbit blood is added. After mixing well the tubes are set in slants. No adjustment of pH.

**Locke-Egg Serum Medium.**—For the cultivation of intestinal protozoa:

1. Take 4 eggs, wash with tap water, brush with alcohol, and carefully break into a sterile flask containing glass beads.

2. Add 50 cc. of Locke's solution.

3. Shake the mixture until the egg is thoroughly broken up.

4. Pour into test tubes a sufficient quantity to produce a slant 1 to 1½ inches in length.

5. Slant the tubes in an inspissator, heat at 70° C. until the mixture is solidified.

6. After complete coagulation, autoclave at 121° C. for 20 minutes, or by fractional sterilization in the Arnold. Care must be taken to raise the temperature in the autoclave very slowly. Care likewise must be exercised to allow the autoclave to cool slowly.

7. Cover the media in the tubes with a mixture prepared as follows: Take equal parts of sterile Locke's solution and sterile inactivated blood serum. Mix and pass through a Berkefeld filter. Incubate the mixture to determine sterility. If sterile, the mixture is ready to be added to the tubes,



## CULTURE MEDIA

## FORMULA OF LOCKE'S SOLUTION

Sodium chloride .....	9.0 gm.
Calcium chloride .....	0.2 gm.
Potassium chloride .....	0.4 gm.
Sodium bicarbonate .....	0.2 gm.
Glucose .....	2.5 gm.
Distilled water .....	1000.0 cc.

Solution is filtered and autoclaved at 121° C. for 15 minutes, and allowed to cool before use.

**Cleveland and Collier's Medium.**—For the cultivation of *Endamoeba histolytica*:

<i>Composition.</i> —Liver-infusion agar (Difco dehydrated) .....	30.0 gm.
Disodium phosphate .....	2.0 gm.
Distilled water .....	1000.0 cc.

*Preparation.*—The medium is placed in tubes, autoclaved at 121° C. for 20 minutes, and slanted. The slants are covered with a 1:6 dilution of sterile fresh horse serum in sterile saline solution. A 5 mm. loop of sterile rice flour or powdered unpolished rice is added to each tube. In making subcultures remove 2 or 3 drops of the rice flour débris from the bottom of the tube with a sterile wide-mouthed pipet.

## GENERAL BACTERIOLOGICAL METHODS

**Principles.**—1. While general bacteriology is an exceedingly complicated subject by reason of the very large number of bacteria known to exist, the number of bacteria producing disease in human beings and the lower animals is comparatively small. The majority of these bacteria are readily detected and identified by present-day methods.

2. Many may be identified by the proper staining of the exudates they produce, supplemented by a study of their cultural and biological characteristics. The technic is relatively simple but demands the employment of accurate methods, including proper methods of staining and differential staining.

3. A good microscope equipped with a satisfactory oil-immersion objective and proper illumination are essential; it is a mistake to temporize with poor objectives, eyepieces and inadequate lighting.

### PREPARATION OF SMEARS OF EXUDATES

1. The examination of stained smears of pus, sputum and other exudates is usually of value in bacteriological examinations and diagnosis; in some instances it constitutes the chief means of diagnosis as in gonorrhea, Vincent's angina, spirofusillar gingivitis, acute contagious conjunctivitis, tuberculosis, leprosy, etc.



FIG. 150.—TOUCHING A HEATED SLIDE TO THE BACK OF THE HAND TO JUDGE THE TEMPERATURE AND AVOID "COOKING"

(From Bass and Jones, *Practical Clinical Laboratory Diagnosis*, The Williams and Wilkins Co., Baltimore.)

2. Slides are preferred to coverglasses as they are less breakable, more easily handled and readily filed. They should be clean and not too badly scratched. Used slides may be used after cleaning as described on page 324.

3. Smears may be prepared with sterile cotton swabs or with flamed stiff wire loops. At least two should be prepared on the same or separate slides.

4. It is important to have smears neither too thick nor too thin. They need not be larger than 1 cm. in diameter if the material is scanty.

5. *Vigorous rubbing should be avoided* as the cells may be broken up and intracellular examinations made difficult or impossible. This is especially important in examinations for gonococci and meningococci or when making a differential count of cells for cytodiagnostics. *The swab should be rolled on the slide* and should not cover the same area twice.

6. Cerebrospinal fluid and other transudates and exudates poor in cells may be first centrifuged and smears prepared of the sediment.

7. In preparing smears of cultures, place a loopful of water on a slide; with a sterile wire transfer a small amount of culture to give an opalescent suspension; spread into a thin layer. The water should be essentially sterile, preferably distilled and not more than 3 days old.

8. Allow smears to dry in the air or with the aid of gentle heating. A slide may be dried by holding it *with the fingers* above a Bunsen flame, since a degree of heat bearable by the fingers will not "cook" or harm the smear (Fig. 150).

9. Do not use the filthy and dangerous method of covering a thick wet smear with another slide.

10. Stained smears keep indefinitely, but if mounted in Canada balsam, cedar oil, or dammar lac they tend to fade unless the preservative is *neutral*.

## METHODS OF CULTIVATING AND ISOLATING BACTERIA AEROBICALLY

1. In transferring bacteria from a culture to a tube or plate of medium, certain precautions must be taken to prevent contamination by outside bacteria. Laboratory air always contains a considerable number of bacteria, and the dust on tables or chairs is full of bacteria. To minimize the chance of contamination, it is advisable to keep tables, chairs and window sills freshly washed. Special "sterile" rooms are often used in which to transfer bacteria. It is also well to keep in mind that when pathogenic bacteria are handled these must not be allowed to escape from the culture and be scattered over the table and into the room. Work as quietly as possible and observe at all times certain precautions and technic.

2. Pasteur transferred bacteria from a culture to a fresh medium by means of a pipet, now known as a "Pasteur pipet." Since the time of Koch the wire needle or loop has been more generally used. These needles are made of platinum or nickel-chromium steel (nichrome, or stainless steel) about 0.025 inch in thickness. The latter is better and also less expensive. The wire needles are sterilized in the flame by heating to red heat. The lower part of the handle should also be sterilized. The Kolle and Rosenberger and Greenman holders are recommended (glass handles are very unsatisfactory because of cracking).

The tubes should be held almost parallel with the table top to avoid air contamination (Fig. 151).

Remove the plugs (do not flame) and hold them between the third and fourth fingers of the right hand; now flame the ends of both tubes (but not too long, as cracking may occur); transfer the material; re flame the ends of the tubes and replace the stoppers. When making smears replace the plugs before spreading the material

on the slide. It is not necessary to flame the stoppers before replacing them. If they are flamed, however, be sure to hold the test tube end of the plug low down in the flame to prevent the loose cotton held by the fingers from catching fire. Be sure that the plugs are inserted so deeply that they will not become loosened. Label properly and preferably with gummed labels as pencil markings may be rubbed off.

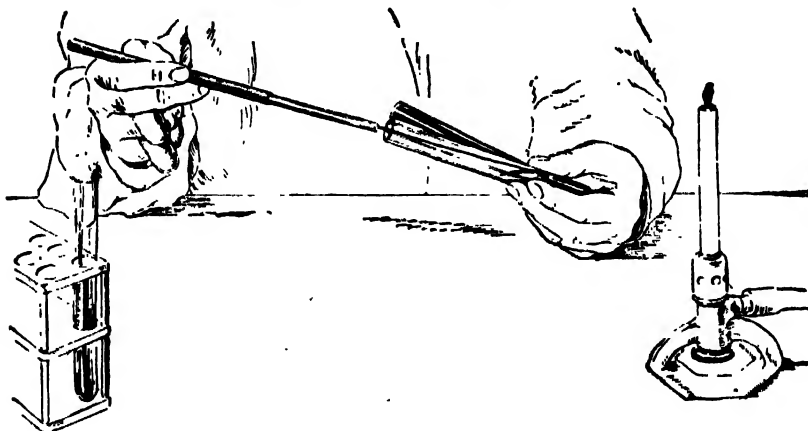


FIG. 151.—METHOD OF HOLDING TUBES AND WIRE FOR INOCULATION OF TUBES  
(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

3. Be careful not to break the surface of a solid medium when inoculating slants. (The butt, however, may be punctured.)

4. If gelatin is being used and if it is somewhat dry, it may be first melted in hot water and allowed to resolidify; inoculate with a deep puncture.

5. If the medium is semisolid, make a deep puncture with a loop, or if a pipet is used, expel the material slowly as the pipet is withdrawn.

6. If the medium is liquid, suspend the material in it with a loop or pipet.

7. When a large amount of fluid material is to be transferred, use a sterile pipet with a cotton plug. When the material is very infectious, attach a piece of rubber tubing with mouthpiece to the pipet or use a Pasteur pipet fitted with a rubber bulb. As soon as the culture has been made, place the pipet in a jar containing a disinfectant. If material is accidentally taken into the mouth, rinse thoroughly with water, then 5 per cent phenol, 40 to 50 per cent alcohol, and again with water.

8. If a Petri plate is to be inoculated, raise the cover at one side just high enough to admit the wire or swab, keeping the plate as completely covered as possible to prevent contamination from the air (Fig. 152).

**Surface Streak Plates.**—Streak plates are made for the purpose of studying colony formations of bacteria and to isolate different bacteria from a mixture.

1. The first essential for making a good plate is to get the proper dilution of the bacteria. Dilutions may be made either in sterile distilled water or in sterile broth. For delicate bacteria the latter is preferred. For making dilutions of various kinds of exudates one may have to rely much on guess work. By examining stained smears of the exudate under the microscope a fair idea can be obtained of the number of bacteria it contains and dilution can be made accordingly. It is, however, simpler to streak a number of plates in series.

2. The streak is best made by the wire loop bent at the tip to form a flat surface about 1 cm. long. The inoculation is made by making 8 or 10 streaks over the surface of the plate. The loop should be allowed to rest lightly when being drawn over the medium to prevent cutting the surface. Where only one plate is streaked, it is best to divide the plate into 4 quadrants and streak in series. If many plates are used, it is very simple to obtain the right dilution.

3. Another good method is to place 1 to 5 loopfuls of the material on the surface of the medium and by means of a bent glass rod, which can be sterilized by flaming, spread the drop over the plate. If many organisms are likely to be present, one or more plates may be smeared without placing any more material on the rod.

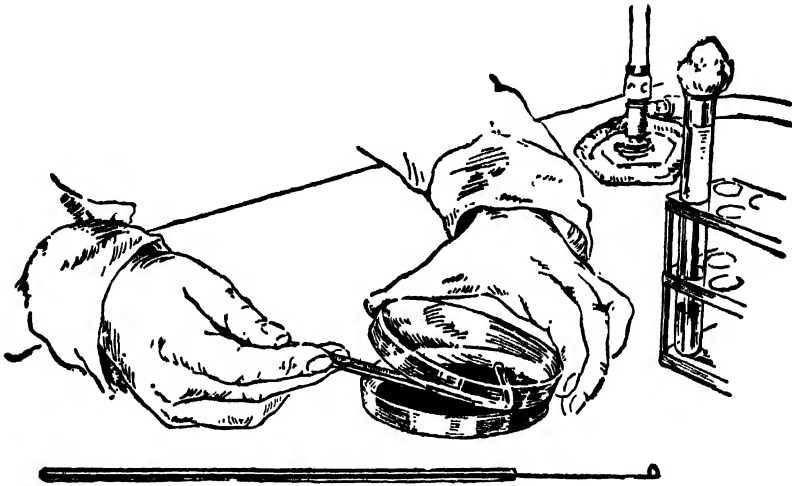


FIG. 152.—METHOD USED IN SPREADING A DROP OF INOCULUM OVER THE SURFACE OF MEDIUM IN A PETRI PLATE BY MEANS OF A STERILE GLASS ROD

(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

4. The surface of streak plates should be fairly dry. A dry surface is obtained by storing for a few days or by using porous porcelain covers, preferably glazed on the outside. The plates should be placed in the incubator upside down.

**Pour Plates.**—Pour plates are made when it is desired to study colonies of bacteria in the agar rather than on the surface, and for the enumeration of bacteria in a culture, or in milk, water, etc.

1. The agar medium in tubes or flasks is melted by immersion in boiling water for a few minutes, or in the Arnold or autoclave. It is then cooled to a temperature between 45 and 50° C. In large laboratories, or when it is desired to have melted agar ready at all times, the method proposed by Magath (*J. Lab. & Clin. M.*, 1928, 13: 672) will be found useful. Two heaters, of about one quart capacity each, are so wired with a switch and lamp that in one position of the switch water is boiled in both containers and, at another position of the switch, the temperature is reduced to about 46° C.

2. For quantitative work, as in the enumeration of bacteria in milk and water, the diluted material is pipetted directly into the sterile Petri dish and the melted agar

at 45° C. is poured on top and mixed (Fig. 153). Generally 10 to 12 cc. of agar is used in each 15 mm. dish.

3. For studying colony formations, as with *blood agar*, the procedure is as follows: To the melted agar at 45 to 50° C. is added 5 to 10 per cent of defibrinated blood (citrate blood is also used). The bacteria, properly diluted, are then added to the blood agar and mixed by rolling the tube between the palms of the hands or by swinging the tube with a circular motion, taking care to prevent foaming of the medium. The inoculated blood agar is then poured into the plate, taking care to flame the mouth of the tube. The drop of agar which generally is left on the mouth of the tube should not be allowed to run down the outside of the tube as it generally will, but should be wiped off with the bottom of the cotton plug. Proper dilutions are usually obtained by putting a loop of the culture into 5 to 10 cc. of sterile water or broth and taking a loop of this into the agar.

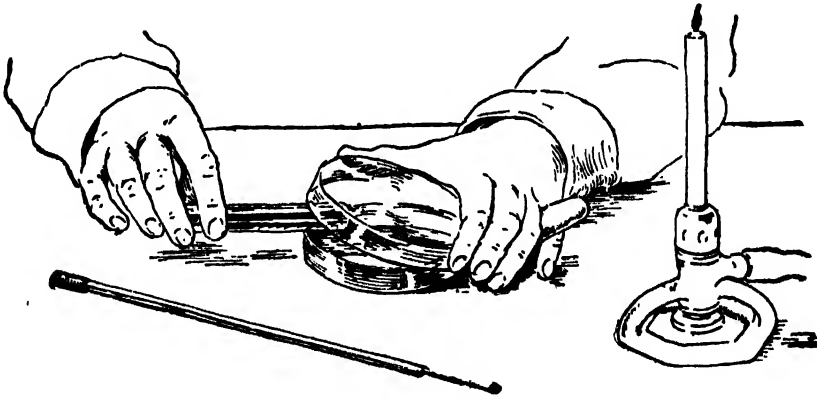


FIG. 153.—METHOD OF POURING INOCULATED MEDIUM INTO A STERILE PETRI PLATE  
(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

4. Or, the following method may be used: Into one of the tubes of melted and cooled agar place a loopful of the material to be examined. Mix well by rotating between the palms of the hands or by very careful shaking to insure uniform distribution. After flaming the wire, transfer 2 loopfuls from this tube to a second and mix thoroughly; repeat by placing 5 loopfuls into the third tube from the second. (This must be carried out rapidly as the agar may solidify before the transfers are completed and thus interfere with the next step.) Pour the agar from each tube into a separate Petri dish, taking care to flame the mouth of each tube and to lift the lids of the plates just enough to admit the end of the tube; distribute evenly by gently rotating and tilting. Allow the agar to solidify, and place the dishes in the incubator, cover side down. If colonies have developed sufficiently at the end of 24 hours, examine both surface and deep colonies on whichever plates they are separated sufficiently to permit of fishing and subculturing. Most of the colonies with this method of plating will be deep and very difficult to differentiate by their growth.

**Isolation of Aerobic Spore-Forming Organisms.**—When material is known or suspected of containing spore-forming bacteria and is likely to be contaminated with other bacteria, a part, suitably diluted with sterile saline or broth, may be heated at 80° C. in a water bath for 30 minutes, or at 70° C. for 1 or 2 hours, to destroy the

nonsporulating or vegetative forms. Then proceed by any of the above methods for isolating pure cultures.

### METHODS OF CULTIVATING AND ISOLATING BACTERIA ANAEROBICALLY

The anaerobic bacteria are those which will not develop unless the oxygen concentration is reduced to a very low value. Such conditions are generally obtained by placing the inoculated tubes or plates in air-tight jars fitted with some system for removing the oxygen. The best method for doing this is either chemically by alkaline pyrogallol, or by combining the oxygen with hydrogen using a catalyst. Deep tubes of broth, especially if they contain particles of meat, etc., or agar freshly boiled, will generally grow most anaerobes. Such tubes of broth are generally covered by a "seal" of petrolatum or a petrolatum-paraffin mixture to decrease the rate of diffusion of oxygen into the medium.

**Shake Tube Method.**—Melt deep agar tubes by placing them in boiling water for several minutes. Cool to about 45° C. and inoculate as directed for making "pour" plates. Allow to harden and incubate. Sufficiently anaerobic conditions are usually found in the deeper layers of medium to cause good growth of most anaerobes. Separate colonies may readily be fished by expelling the agar into a sterile Petri dish. This is best done by means of a pipet similar to a Pasteur pipet which is made from glass tubing. The tip of the pipet should be drawn rather thin and the end sealed in the flame. Gently heat the bottom of the tube in the flame to melt the agar slightly and to loosen it. Push the tip of the pipet through the agar and hard against the bottom of the tube so that the end of the pipe is broken. Now blow through the pipet and withdraw it as the agar is pushed from the tube. By means of the pipet the agar is transferred to a sterile Petri dish. With a sterile wire the agar may be cut in any desired place to expose the colonies desired and permit fishing.

**Liquid Tubes with Petrolatum Seal.**—The medium is preferably freshly boiled to drive out the oxygen. It is then allowed to cool in a slanting position. Since it is impractical to inoculate the medium through the petrolatum seal it is best to make an opening in the seal. This is best done by gently warming the bottom of the slanted seal which then flops up leaving a clear opening into the medium. When inoculated the petrolatum is gently warmed and allowed to cover the medium.

**Liquid Tubes with Marble Seal (Hall).**—Special tubes are used with a constriction below the middle. In this constriction is placed a glass ball or marble. The medium below this seal is generally quite free of oxygen.

**Alkaline Pyrogallate Methods.**—The oxygen may be absorbed by alkaline pyrogallol in individual agar tubes as in the so-called *Wright tube*. The agar in such tubes may be inoculated either as stabs or slants, usually the latter. The cotton plug is clipped off with a pair of scissors and pushed into the tube for a short distance. On top of the plug is then placed a small quantity of pyrogallic acid and sodium carbonate. Just before inserting a tight-fitting rubber stopper, a small quantity (1 to 2 cc.) of water is poured in and the rubber stopper pushed in tight. A small quantity of sodium hydroxide may be used instead of the carbonate and water. The tube should be incubated with butt end up.

**Spray Plate Method.**—This consists of a modification of the McLeod method and employs a specially designed plate made of pyrex glass consisting of the bottom of a Petri dish and a special dish with an impressed ridge on the bottom and a rolled moat around the top (*J. Lab. & Clin. M.*, 1930, 17: 203).

1. These 2 parts are assembled, wrapped with paper and sterilized by baking.
2. Pour the inoculated blood agar or other medium into the Petri dish and immediately invert the bottom of the apparatus over it while the agar hardens. Hardened plates may be surface streaked.

3. Invert; raise the Petri dish and on one side of the ridge place 4 cc. of 40 per cent pyrogallic acid and on the other side 10 cc. of 20 per cent sodium hydroxide. Replace the Petri dish and seal by pouring around the moat, hot paraffin, a mixture of 2 parts paraffin and 1 part beeswax or a good grade of oil clay. One may seal in a string with the ends protruding from the paraffin or clay to aid in opening the dish later.

4. Tip the dish to mix the solution and incubate.

5. The plate may be examined repeatedly without opening and when proper growth has developed the plate is pried off or the string pulled to cut the seal and the colonies examined.

**Brewer Plate Method.**—The B.B.L. anaerobic agar is recommended (page 354), the method being as follows:

1. The sterile medium is melted, cooled to 50° C. and distributed in about 40 cc. amounts if sterile 15 mm. Petri dishes are used and in 25 cc. amounts if 10 mm. dishes are employed. The former are preferred as they may be incubated for longer periods without drying out. It is essential that the depth of the medium in the dish should be sufficient for the rim of the anaerobic cover to rest on the surface of the agar and not on the Petri dish at any point.

2. Allow to harden. A porous cover is preferred.

3. The medium may be inoculated before pouring or by streaking the central portions.

4. After the agar has solidified replace the Petri dish cover by the Brewer anaerobic lid which is so designed that it touches the agar at the periphery and traps a small amount of air, less than 1 mm. in thickness, over the surface of the agar (Fig. 154). The reducing agent in the medium uses up the oxygen in this small amount of air and an anaerobic condition develops. The glass rim on the lid forms a seal with the moist agar and no other seal is necessary. The methylene blue in the agar acts as an indicator and the center of the dish which is anaerobic becomes colorless, while there is a blue oxygenated edge of the plate about 5 mm. in diameter.

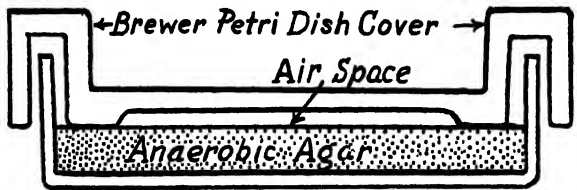


FIG. 154.—BREWER PETRI DISH COVER FOR ANAEROBIC CULTIVATION

**Anaerobic Jar Methods.**—1. First heat the medium in boiling water for 15 to 20 minutes to drive off oxygen and cool to 42 to 45° C. before inoculating. Incubate in a



jar from which the oxygen has been removed. Any large jar with a tight fitting cover or stopper may be made into an anaerobic jar by the use of alkaline pyrogallate.

The *Novy jar* (Fig. 155) is an example of a jar in which the oxygen is displaced by hydrogen or other inert gases such as nitrogen. These jars are generally first evacuated, then filled with hydrogen, again evacuated, and finally filled with hydrogen.

The *McIntosh and Fildes jar* illustrates the type of jar in which the oxygen is removed by combination with hydrogen by means of a platinum-block catalyst (Fig. 156).

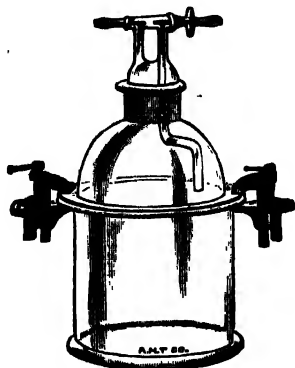


FIG. 155.—IMPROVED NOVY ANAEROBIC CULTURE APPARATUS

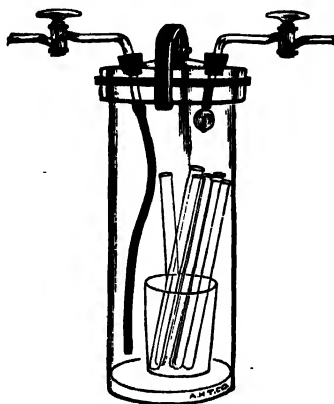


FIG. 156.—SMILLIE ANAEROBIC CULTURE APPARATUS

The *Weiss-SpaULDing* apparatus shown in Figure 157 is satisfactory and can be readily set up. Cultures are placed in a sealed Hempel desiccator with 0.5 gm. pal-

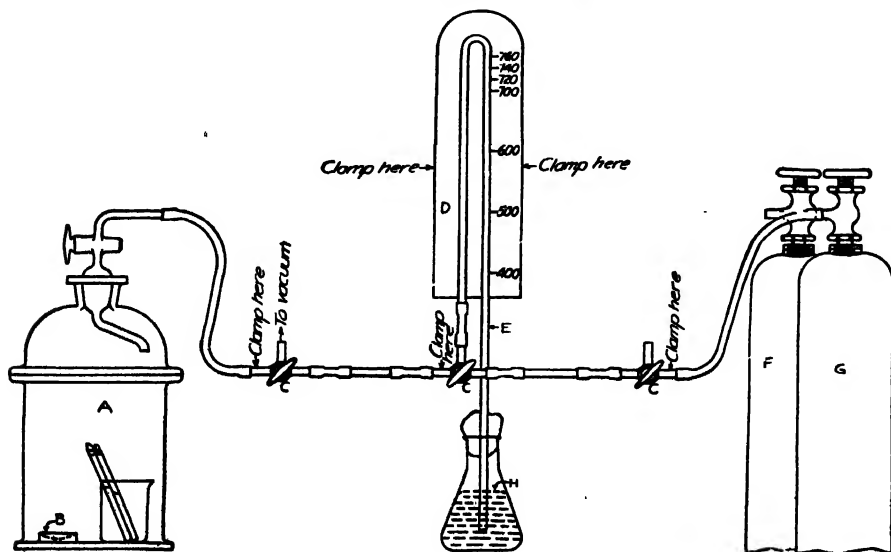


FIG. 157.—THE WEISS-SPAULDING APPARATUS FOR ANAEROBIC CULTURES

A, Hempel desiccator; B, catalyst in jar; C, three-way cocks; D, mirror for reading column of mercury; E, mercury manometer; F, tank of carbon dioxide; G, tank of hydrogen; H, reservoir of mercury.

ladinized asbestos (shredded) and a tube of 2 per cent dextrose in nutrient broth colored with methylene blue (1:50,000). The air is exhausted from the jar by suction and the stop-cock closed. Carbon dioxide is then introduced to a manometer reading of 700 mm. mercury. Hydrogen is then slowly introduced until atmospheric pressure is reached. The stop-cocks are then closed and the jar disconnected for incubation of the cultures.

The *Brown jar* is also quite satisfactory. The catalyst in this jar is platinized asbestos placed on a glass rod and wrapped around with resistance wire through which is sent a current of electricity to heat the catalyst. The catalyst is enclosed in a netting of fine wire to prevent explosion.

The inoculated cultures (Petri dishes or tubes) are placed within the jar. A piece of "plasticine" (other modeling clays have been found less satisfactory) is rolled out in the form of a thin roll and placed onto the ground edge of the glass jar. The lid is pressed down onto the plasticine so as to make an air-tight joint and is held in place by the screw clamp. If a vacuum pump is available, a large part of the air may be pumped out of the jar. This serves to draw the lid down into place, to minimize the danger of explosion, and to establish anaerobic conditions more quickly. With the partial vacuum in the jar it is connected with the source of hydrogen which is led into the jar under a pressure of only 2 or 3 pounds. The terminals of the heating coil having been connected with an ordinary 110-volt electric light current which has been reduced by passage through a 50-watt light bulb, the current is turned on and allowed to flow for 20 or 30 minutes. If the connections are tight and the hydrogen tank is provided with a reducing valve, the hydrogen may be allowed to flow into the jar throughout this period as fast as it is consumed, a pressure of 2 or 3 pounds being maintained by the reducing valve. When combustion is complete, the hydrogen inlet is closed, the electric current turned off and disconnected, and the jar is ready to be incubated. When used in this manner the atmosphere within the jar consists almost wholly of hydrogen. If it is desired to retain an atmosphere composed of nitrogen and a lesser amount of hydrogen, the vacuum pump need not be used before the hydrogen is admitted and the electric current is turned on.

**Method of Cultivating Bacteria under an Increased  $\text{CO}_2$  Tension.**—This method is used for the isolation of the bovine type of *Brucella* and often for meningo-

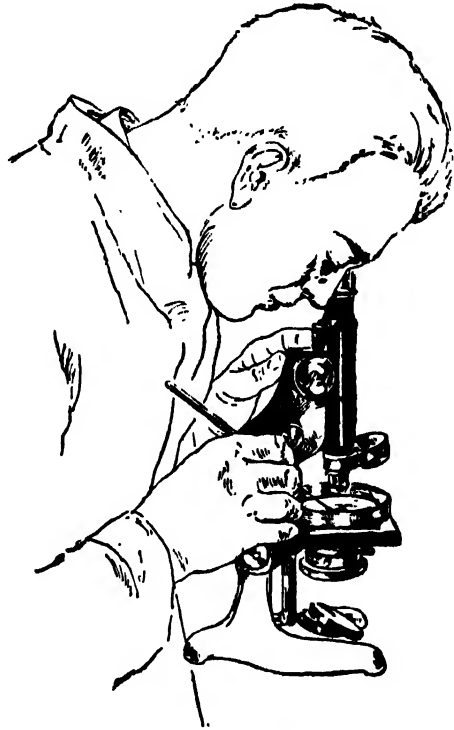


FIG. 158.—FISHING AND TRANSFERRING A COLONY WITH THE AID OF THE MICROSCOPE

(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

cocci and gonococci. The best concentration of  $\text{CO}_2$  seems 5 to 10 per cent. An anaerobic jar such as that of Weiss and Spaulding is most convenient to use. Connect the jar with a vacuum pump by means of a three-way stop-cock which is also connected with a mercury manometer and a source of  $\text{CO}_2$ . Remove sufficient air to decrease the pressure by 5 cm. of mercury and then replace this air with carbon dioxide.

**Methods for Fishing Colonies.**—Unless well-isolated colonies are found on a plate it is best to replate if possible. Examinations of the colonies may be facilitated by means of a hand lens, dissecting microscope or the low power of the microscope. The colony selected for fishing should be "ringed" with a wax pencil and numbered. Only a part of the colony should be removed for staining, leaving the rest for transfer to sterile media as desired. The fishing is best done with a sharply pointed needle. Colonies in shake tubes are best fished by first expelling the agar as described under Shake Tube Method. The agar is then cut with a sterile knife or wire close to the desired colony.

### MICROSCOPICAL EXAMINATION OF CULTURES AND EXUDATES

1. For smears of cultures on solid media place a small loop of water on the clean slide. With the needle add a minute amount of growth to the water. Mix, spread and dry in the air. From fluid media spread small loop of culture onto the slide; no water need be used. When the smear is perfectly dry "fix" it by passing the slide back and forth through the flame three times (do not overheat) and allow it to cool before staining. For special purposes fixing may be done with methyl alcohol or other reagents.

2. In the case of Petri dishes, remove the cover and place it right side up on the table.

3. Examine the plate with unaided eye or hand lens and ring off selected colonies with wax pencil on bottom of plate.

4. Or select colonies with the aid of the lower part of the microscope (Fig. 158).

5. With a sterile needle carefully remove portions of selected colonies to fresh media and prepare smears for staining. With adherent colonies and especially pneumococci and streptococci, it is sometimes necessary to cut out with a sterile loop a portion of the medium and transfer to broth.

6. If the colonies are not well isolated, fish several to broth and prepare another set of plates.

**Moist Preparations.**—For examining unstained material suspend some of the material in a small drop of water, salt solution, broth or mounting fluid<sup>1</sup> on a clean glass slide. Carefully, so as not to admit air bubbles, place a clean coverslip over the drop. Examine with any of the objectives of the microscope required but it will be found necessary to reduce the light by partially closing the diaphragm of the microscope to obtain clear definition of unstained bacteria and other cells. Moist preparations are to be dropped into boiling water or into disinfecting solution before being cleaned as a safeguard against accidental infection.

**Hanging-drop Preparations.**—In the center of a clean thin (No. 1) coverslip 20 mm. square place a small loop of material to be examined (fluid culture or sus-

<sup>1</sup> Mounting fluid, especially useful for examination of fungi, is prepared by mixing equal volumes of glycerol, ammonium hydroxide and alcohol.

pension of growth from solid medium). Do not spread the droplet. Place small bits of vaselin or droplets of oil on opposite sides of the concavity of a hollow-ground slide (Fig. 159). Invert the coverslip over the concavity of the slide so that the drop of material hangs beneath the coverslip without touching the slide into the concavity and with opposite corners of the coverslip protruding beyond the edges of the slide (to facilitate removal of the coverslip when it is to be discarded). Hanging-drop preparations are to be dropped into boiling water or into disinfecting solution before being cleaned. It is not easy to focus the microscope on a hanging drop. Reduce the light with the diaphragm and find the edge of the drop under the low power objective, then turn the high power objective into place. (It is important that the objectives of the microscope be par-focal and accurately centered). Having found the edge of the drop under high power, the slide may be moved about carefully while under observation of the technician.



FIG. 159.—HANGING DROP SLIDE

**Darkfield Examination.**—1. Apparatus necessary (Fig. 160); strong illuminating lamp such as a small arc lamp or high-power incandescent lamp; funnel stop to be



FIG. 160.—OUTFIT FOR DARKFIELD EXAMINATION FOR SPIROCHETES  
(From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

placed in oil-immersion objective to cut out rays which interfere, and a special substage condenser (Fig. 161).

2. Remove substage condenser from microscope and adjust darkfield condenser in its place.

3. Insert funnel stop in the oil-immersion objective if one is to be used. One may use a special oil-immersion lens of N.A. 0.80 without a funnel stop.

4. Center the apparatus with low-power lens by getting concentric rings on the upper surface of the darkfield condenser and adjusting by means of the three centering screws on edge of the condenser until the rings are parallel with the circle of the microscopic field.

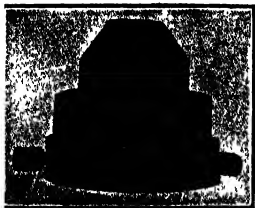


FIG. 161.—SUBSTAGE CONDENSER FOR DARKFIELD EXAMINATION

5. Place a small drop of material to be examined on a scrupulously clean slide (1.45 to 1.55 millimeters thick) and cover with clean coverglass (avoid air bubbles).

6. Place large drop of oil on top of condenser and put slide on stage so the oil forms a contact with the under surface of the slide.

7. Place oil on top of coverslip and examine with oil-immersion objective. Examination can also be made with low and high power objectives. A darkened room is helpful.

### METHODS OF STAINING BACTERIA

Stains are most conveniently kept in dropping bottles or bottles provided with a rubber stopper and nipple with a short dropping pipet attached. The staining is generally done by putting the stain on the slide, but for some purposes a staining dish is employed.

Only sufficient stain for covering the smear should be used in the interests of economy. It should not be spread with the tip of the bottle as contamination of the stain may result.

**Carbolfuchsin for General Staining.**—This is an excellent stain for bacteria in general.

1. Fix the smear with heat and cover with water.
2. Add a drop or two of Ziehl-Neelsen's stain or cover smear with a 1:10 dilution.
3. Stain for 30 to 60 seconds, wash with water, dry and examine. Bacteria are of a deep pink color. It is important not to overstain.

**Gram Stain.**—1. Prepare smears as thinly and uniformly as possible and fix by heating.

2. Stain the preparation for 15 to 30 seconds with a 2 per cent solution of crystal violet in absolute (C.P.) methyl alcohol. This stain is much more satisfactory than the unstable aniline gentian violet originally used.

3. Wash with water.

4. Apply Gram's iodine solution for 1 minute. This solution is prepared by dissolving 1 gm. of iodine and 2 gms. of potassium iodide in 300 cc. of distilled water.

5. Wash with water.

6. Decolorize with 95 per cent alcohol until washings have only a slight violet color (Fig. 162). Acetone, suggested by Lyon, has come into extensive use, in place of alcohol, as a decolorizer but decolorizes very rapidly. A mixture of 2 parts alcohol and 1 part of acetone is satisfactory. As shown by Spray it is important to decolorize with alcohol only when staining the *Clostridia* of gangrene.

7. Wash with water and counterstain with a 1 per cent aqueous solution of safranin for 10 seconds. A 1:10 dilution of Ziehl-Neelsen's carbolfuchsin for 10 seconds is also satisfactory. Pappenheim's pyronine-methyl-green stain if properly made is also

satisfactory as it brings out gram-negative bacteria sharply, and is especially desirable for intracellular gram-negative organisms like the gonococcus, meningococcus and influenza bacillus (organisms bright red and nuclei of cells blue).

8. Wash with water. *Do not blot* but allow to dry by standing slide on end against a solid object. Drying may be hastened by first shaking off excess water and then passing the slide a few times through a flame.

9. If, with a proper technic, gram-positive organisms are decolorized, the fault probably lies in the iodine solution, which tends to become acid when long exposed to light. It can be corrected by adding a pinch of sodium bicarbonate to the bottle of solution.



FIG. 162.—METHOD OF DECOLORIZING IN GRAM STAIN

(From Bass and Jones, *Practical Clinical Laboratory Diagnosis*, The Williams and Wilkins Co., Baltimore.)

The *Weiss modification of the Gram stain* (*Jour. Lab. and Clin. Med.*, 26:1518, 1941) is as follows: 1. Prepare smears as thinly and uniformly as possible and fix them over the flame.

2. Cover the slide with 3 per cent gentian violet (3 gm. of gentian violet, 20 cc. of 95 per cent alcohol and 80 cc. of distilled water) for 3 to 5 minutes.

3. Wash with warm water.

4. Cover with 20 times concentrated Gram's iodine solution (20 gm. of iodine, 40 gm. of potassium iodide and 300 cc. of distilled water) for 3 to 5 minutes.

5. Wash with warm water.

6. Cover (decolorize) the slide with acetone and wash off immediately with water.

7. Counterstain briefly (on and off) with a 1:4 aqueous dilution of a 2 per cent solution of basic fuchsin in 95 per cent alcohol.

8. Wash with water and allow to dry.

*Burke's modification of the Gram stain* is as follows: 1. Make thin smear on glass slide or coverglass.

2. Dry in air and fix with gentle heat.

3. Cover smear with 1 per cent aqueous solution of gentian violet.

4. Immediately mix the dye on the slide with 3 to 5 drops of a 5 per cent solution of sodium carbonate in a 0.5 per cent aqueous solution of phenol.

5. Stain for 1 to 2 minutes.

6. Wash quickly with water and cover for 1 minute with Gram's iodine solution.

7. Wash quickly with water and blot.

8. Decolorize by dropping a mixture of 3 parts acetone and 1 part ether until the solution flows colorless from the slide. Equal parts of acetone and 95 per cent alcohol or 2 parts alcohol and 1 part of acetone are equally satisfactory. Blot dry.

9. Counterstain for 30 seconds with a 0.5 per cent aqueous solution of safranin.

10. Wash with water, blot and dry. Gram-positive organisms are very dark blue; gram-negative organisms are an orange pink.

*Hucker's modification of the Gram stain* is as follows:

#### SOLUTION A

Crystal violet (85 per cent dye content) . . . .	3.0 gms.
Ethyl alcohol (95 per cent) . . . . .	20.0 cc.

#### SOLUTION B

Ammonium oxalate . . . . .	0.8 gm.
Water . . . . .	80.0 cc.

Mix solutions A and B, ordinarily in equal parts. It is sometimes found, however, that this gives so concentrated a stain that gram-negative organisms do not properly decolorize. To avoid this, solution A may be diluted as much as ten times, and 20 cc. of the diluted solution mixed with solution B.

1. Stain 15 seconds with the gentian violet solution.

2. Wash in water.

3. Immerse in Gram's iodine solution for 1 minute.

4. Wash in water and blot dry.

5. Decolorize with 95 per cent alcohol or acetone (preferred) for 5 to 10 seconds with gentle agitation.

6. Drain off and counterstain for 10 seconds.

7. Wash, dry, and examine.

**Löffler's Methylene Blue.**—For general staining of bacteria and for diphtheria bacilli:

1. Make thin smear of material to be examined on slide.

2. Dry in air and fix with gentle heat.

3. Cover smear with stain and allow to stand for 1 minute: heat slightly if deep staining is desired.

## FORMULA OF STAIN

Methylene blue (certified *)	0.3 gms.
Ethyl alcohol (95 per cent)	30.0 cc.
When dissolved add	
Distilled water	100 cc.

4. Wash with tap water, blot and examine with oil-immersion lens.

Methylene blue does not stain very intensely and there is little danger of over-staining. It is a good stain to use when studying the morphology of organisms and is sometimes used in the examination of cultures for diphtheria bacilli.

**Borax Methylene Blue (Manson).**—For staining bacteria in blood smears. Same technic as above:

## FORMULA FOR STAIN

Methylene blue	2 gms.
Borax	5 gms.
Distilled water	100 cc.

**Wayson Stain.**—For staining diphtheria bacilli and to show nuclear structures for bacteria:

1. The stain is prepared by dissolving 0.2 gm. of fuchsin and 0.75 gm. of methylene blue in 20 cc. of absolute alcohol. Add the dye solution to 200 cc. of 5 per cent solution of phenol in distilled water. Filter.

2. Stain smears for a few seconds. Wash, blot and dry.

**Neisser's Stain for Diphtheria Bacilli.**—1. Prepare smear in usual manner, fix with heat and place in solution No. 1 for 2 or 3 seconds.

## SOLUTION NO. 1

Methylene blue (Grubler)	0.1 gm.
Alcohol (95 per cent)	2.0 cc.
Glacial acetic acid	5.0 cc.
Water (distilled)	95.0 cc.

Dissolve the methylene blue in alcohol and add to the acetic acid and water.

2. Wash in tap water.

3. Place in solution No. 2 for 3 to 5 seconds.

## SOLUTION NO. 2

Bismarck brown	0.2 gm.
Water (boiling)	100.0 cc.

Dissolve the stain in boiling water and filter through filter paper.

4. Wash, dry, and mount.

5. The bacilli may stain uniformly brown or may show at one or both ends a

\* The term "certified" as applied to stains indicates a product which has been found satisfactory by the Commission on Standardization of Biological Stains of the Society of American Bacteriologists.

Löffler's methylene blue stain was prepared by adding alkali to the solution. Modern purified samples of methylene blue do not require this. The older preparations contained acid impurities (Conn; Stain Tch., 1929, 4:27. Conn; Biological Stains, Geneva, N. Y., 1929, 67).



dark blue, round body. True diphtheria bacilli usually show the blue bodies, while the pseudotypes show few if any.

**Albert's Stain (Laybourn's Modification for Diphtheria Bacilli).**

**SOLUTION NO. 1**

Toluidin blue .....	0.15 gm.
Malachite green .....	0.20 gm.
Glacial acetic acid .....	1.00 cc.
Alcohol (95 per cent) .....	2.00 cc.
Distilled water .....	100.00 cc.

Let stand 24 hours, filter.

**SOLUTION NO. 2**

Iodine crystals .....	2 gm.
Potassium iodide .....	3 gm.
Distilled water .....	300 cc.

1. Fix smears by heat.
2. Flood with solution No. 1 for 3 to 5 minutes.
3. Wash in tap water.
4. Flood with solution No. 2 for 1 minute.
5. Wash, blot dry, and examine.
6. The granules stain an intense black, the bars dark green, and the intervening portions light green. The stain is quite specific and will serve to detect diphtheria bacilli when only a few are present.

**Carbolfuchsin (Ziehl-Neelsen).**—For staining “acid-fast” bacteria (tubercle bacilli, leprosy bacilli, etc.):

1. Make thin smear on a slide or coverglass. Dry in air and fix with heat.
2. Flood the smear with the following stain and steam gently over the flame about 3 minutes. Do not boil and renew the stain as it evaporates:

10 per cent alcoholic solution of basic fuchsin ..	10 cc.
5 per cent aqueous solution of phenol .....	100 cc.

3. Wash with water and decolorize by dropping acid alcohol on the smear until it flows colorless from the slide:

Concentrated hydrochloric acid .....	2 cc.
70 per cent ethyl alcohol .....	98 cc.

4. Wash with water and counterstain with Löffler's methylene blue for 1 minute.
5. Wash with water, blot, dry and examine. “Acid-fast” bacilli are pink in a blue background.

**Gabbet's method.**—For staining tubercle bacilli.

1. Prepare a thin and even smear on a slide or coverglass. Dry in air and fix with heat, or by immersion in a 1 per cent aqueous solution of bichloride of mercury for 2 or 3 minutes; fixation in methyl alcohol for 2 or 3 minutes is also satisfactory.

2. Cover with Ziehl-Neelsen carbolfuchsin and steam for 3 minutes, replacing the stain with a dropper as it evaporates. Too great heat will interfere with the staining of some of the bacilli, probably by destroying the waxy substance upon which their acid-fastness depends.

3. Wash in water.

4. Apply Gabbet's stain for 15 to 30 seconds. The stain consists of 2 gm. of methylene blue dissolved in 75 cc. of distilled water and 25 cc. of concentrated sulfuric acid.

5. Wash in water. If the thinner portions of the smear are still red, apply Gabbet's stain for another 15 to 30 seconds and wash with water. Too long application of the stain may decolorize tubercle bacilli.

6. Blot with filter paper and warm over the flame until thoroughly dry.

**Pappenheim's Method.**—For the staining of acid-fast organisms.

1. This is the same as Gabbet's method, except that Pappenheim's methylene blue solution is substituted for Gabbet's stain and is allowed to act for 1 to 2 minutes. The stain is prepared by dissolving 1 gm. corallin (rosolic acid) in 100 cc. of absolute ethyl alcohol, adding 20 cc. of glycerol and as much methylene blue as will go into solution (saturated). To avoid evaporation the stain must be kept in a tightly stoppered bottle.

2. The method is very satisfactory for routine work as decolorization of tubercle bacilli is practically impossible. The stain was originally recommended as a means of differentiating smegma from tubercle bacilli, the former being decolorized, but it is not to be relied upon for this purpose.

**Richards and Miller Fluorescent Dye Method.**—This method described by Richards and Miller (*Am. Jour. Clin. Path. Tech. Suppl.*, No. 5, 11: 1, 1941) has proven very satisfactory for the detection of tubercle and leprae bacilli although it necessitates the use of a darkened room, smears stained with a fluorescent dye (Auramine O) and ultra-violet light.

1. Prepare a smear in the usual manner on a slide. Fix with heat.

2. Apply the stain for 2 or 3 minutes. The stain may be prepared as described by Thompson (*Proc. Staff Meet. Mayo Clinic*, 16: 673, 1941) as follows:

#### SOLUTION A

Distilled water .....	87 cc.
Liquefied phenol .....	3 cc.
Mix thoroughly.	

#### SOLUTION B

Ethyl alcohol (95 per cent) .....	10 cc.
Auramine O (Nat. Aniline Co.) .....	0.1 gm.
Add Solution B to Solution A.	

3. Wash in tap water.

4. Apply decolorizing solution for 2 or 3 minutes. Pour off and apply the solution again for another 2 to 5 minutes. The decolorizing solution is composed of 100 cc. of 70 per cent ethyl alcohol, 0.5 cc. of concentrated hydrochloric acid and 0.5 gm. of sodium chloride. If the smear is quite thick it is advisable to double the amount of hydrochloric acid in the decolorizing solution and to bleach for a total of 10 minutes. Good decolorization is essential. An incompletely stained yellowish background lowers the visibility of the organisms. Prolonged decolorization, even over night, does not bleach the bacteria.

5. Wash in water and dry.

6. Examine with ordinary microscope using an 8-mm. objective with a  $20\times$  ocular with accessories as illustrated in Figure 163. Set up the microscope with the yellow filter resting on the diaphragm inside the ocular. Place the aluminized mirror over the microscope mirror and the blue, ultra-violet transmitting filter over the microscope lamp. It is convenient to focus the light before the blue filter is placed on the lamp. The microscope condenser should be opened completely after the blue filter is placed on the lamp.

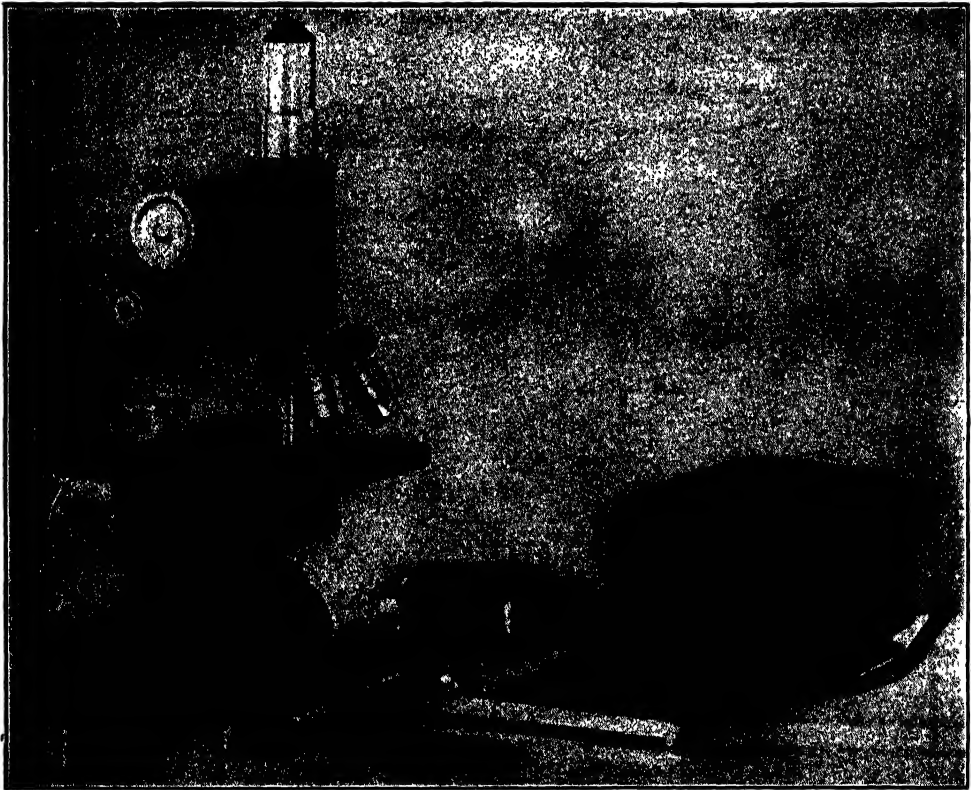


FIG. 163.—ATTACHMENTS FOR FLUORESCENT METHOD OF EXAMINATION FOR ACID FAST ORGANISMS

A, Yellow filter to go into ocular; B, blue, ultraviolet transmitting filter for lamp.  
(Courtesy of Spencer Lens Company, Buffalo, New York.)

The room should be darkened as the bacilli appear as bright, luminous yellow rods against the black background (Fig. 164). Because they are self-luminous they appear a little longer than usual. If the room is bright, extraneous light should be shielded from the examiner by a black cloth or screen between the microscope and the interfering light. It may take a short time for the eye to become sufficiently adapted to see the bright yellow organisms. When large numbers of slides are to be examined, a screen covering three sides of the microscope to exclude light is desirable.

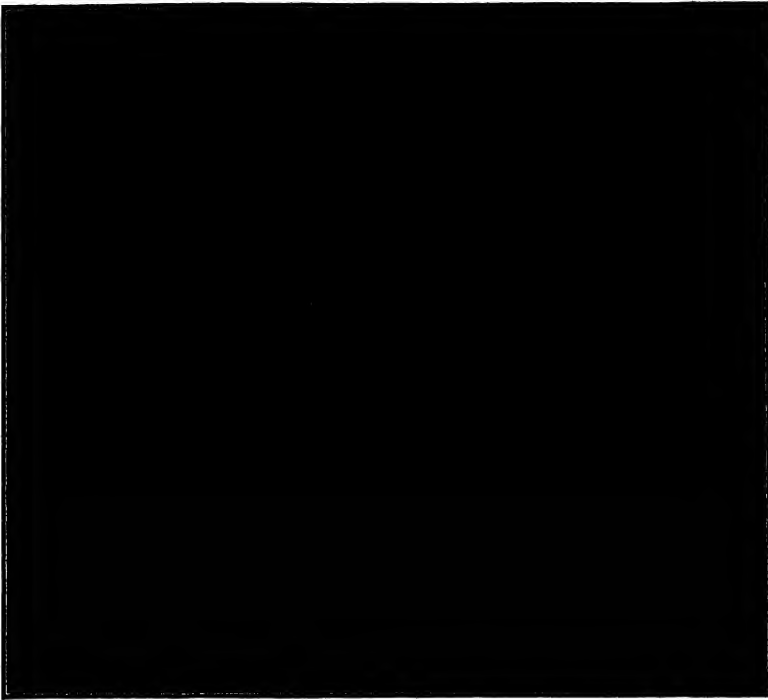


FIG. 164.—FLUORESCENT TUBERCLE BACILLI IN SPUTUM  
(Courtesy of Spencer Lens Company, Buffalo, New York.)

### Flagella Stain (Leifson, Modified).

#### FORMULA

Potassium alum, 5 per cent aqueous solution....	10 cc.
Tannic acid, 2 per cent aqueous solution.....	10 cc.
Basic fuchsin (dye content 100 per cent) in 1 per cent solution in 95 per cent alcohol.....	10 cc.
The solutions are mixed in the order given.	

The tannic acid solution usually develops molds and for this reason will not keep. A mixture of solutions (1) and (2) seems to keep and also seems to be stable. Rosaniline hydrochloride may be used instead of the basic fuchsin. The concentration given is based on the pure dye and since the commercial dyes are seldom pure (75 to 90 per cent) they must be used in correspondingly higher concentration. For example, a dye labeled "85 per cent pure" should be used in 1.18 per cent concentration.

The flagella stain is stable for several days provided it is kept well stoppered. A new mixture need only be made about once a week. The precipitate which forms is of no consequence. In old stains it is best to use only the supernatant.

1. Use only young broth or moist agar cultures. Both cultures are centrifuged, the supernatant fluid poured off, and the bacteria carefully suspended in distilled water. The suspension is again centrifuged, the supernatant poured off, and the bacteria re-suspended in distilled water. Smears from agar slants are made by suspending some of the growth from the bottom or middle of the slant in distilled water. The growth

at the top of the slant should not be used. Place a large loopful of the suspension on the end of a prepared slide. Tilt the slide to cause the suspension to flow down the slide. If it does not flow readily the slide is greasy and should be discarded. Allow to dry in air at room temperature. No fixing is necessary.

2. Slides should be free from scratches and absolute grease-free. The best method of cleaning the slides is to put them in a hot solution of potassium dichromate in concentrated sulphuric acid for a few hours. They are then thoroughly washed with distilled water and allowed to drain and dry in air. They should never be wiped with a cloth. Just before use the slide should be flamed strongly (film side towards the flame), and a wax pencil line made along the edges of the slide leaving about  $\frac{1}{2}$  inch free for handling. The wax pencil lines serve to confine the stain and allows it to be heaped up on the slide.

3. With a capillary (Pasteur) pipet place 0.8 to 1 cc. of the stain on the prepared slide. The slide should be level or on a very slight incline. It is important that the stain be heaped up on the slide. Allow the stain to remain on the slide for about 10 minutes. With a high room temperature and much circulation of air the staining may be complete in about 5 minutes. In a cold room with no circulation the staining may take 15 minutes. The staining of the flagella depends upon the evaporation of the alcohol and consequent formation of a colloidal precipitate of the dye complex which is adsorbed by the flagella.

4. Wash with water and examine, or counterstain if desired.

5. Counterstaining is not necessary for routine work. With some bacteria such as typhoid bacilli, the body of the organism is stained only faintly or not at all and the counterstain helps to give a better picture. A satisfactory counterstain is made by diluting borax methylene blue 10 times with distilled water (0.1 per cent methylene blue and 0.5 per cent borax). Allow counterstain to act about 10 minutes. By this means the bacterial bodies become blue and the flagella red.

**Löffler's Method for Flagella.**—1. Prepare slides or coverglasses as described above.

2. Prepare smears of young cultures as described above.

3. Fix by heating over a flame while holding in the fingers.

4. Cover with freshly filtered mordant and gently warm for about a minute. The mordant consists of 10 cc. of a 20 per cent aqueous solution of tannic acid, 5 cc. of a cold saturated aqueous solution of ferrous sulfate and 1 cc. of a saturated aqueous or alcoholic solution of gentian violet. Wash in water.

5. Apply freshly filtered aniline-gentian-violet, warming gently for 30 to 60 seconds. The stain (Stirling's) is prepared of 5 gm. gentian violet, 10 cc. ethyl alcohol, 2 cc. aniline oil and 88 cc. of distilled water.

6. Wash in water, dry, and mount in balsam.

**Casares-Gil Method for Flagella.**

#### MORDANT

Tannic acid .....	10	gms.
Aluminum chloride ( $\text{Al}_2\text{Cl}_6 \cdot 12\text{H}_2\text{O}$ ) .....	18	gms.
Zinc chloride .....	10	gms.
Rosaniline hydrochloride .....	1.5	gms.
Alcohol (60 per cent) .....	40	cc.

The solids are dissolved in the alcohol by trituration in a mortar, adding 10 cc. of the alcohol first and then the rest slowly. This alcoholic solution may be kept several years.

1. To make the preparation of bacteria: Transfer 1 loopful of growth from an 18-hour agar slant culture of the organism to 2 cc. of sterile tap water. Incubate this suspension at 37° C. for 10 to 15 minutes. Transfer one loopful carefully to an absolutely clean slide. Tilt the slide so that the drop runs down, leaving a thin film, or the drop may be drawn out gently with paper. Allow to dry in air.

2. Dilute the mordant with 2 parts of distilled water, filter off the precipitate and collect the filtrate on the slide containing the smear of bacteria. Allow this to act 1 to 2 minutes. A precipitate and metallic sheen should form. Wash with distilled water.

3. Cover preparation with filtered carbolfuchsin and allow to act 1 to 3 minutes. Wash in distilled water. Dry without blotting.

**Dorner's Method for Spores.**—*Solutions.*—(a) Carbolfuchsin (freshly filtered); (b) saturated aqueous solution of nigrosin B (Grübler).

1. Make a heavy suspension of the organism in 2 to 3 drops of distilled water in a small test tube. Use the growth of the culture on an agar slant for this emulsion.

2. Add an equal quantity of freshly filtered carbolfuchsin.

3. Allow the mixture to stand in a boiling water bath 10 to 12 minutes.

4. On a coverslip or slide mix 1 loopful of the stained preparation with 1 loopful of a saturated aqueous solution of nigrosin.

5. Smear as thinly as possible and dry rapidly.

The spores are stained red, the bodies of the bacteria are almost colorless and stand out against the dark gray background of nigrosin.

**Moeller's Method for Spores.**—1. Coverslips are prepared as usual and fixed in the flame.

2. Wash in chloroform for 2 minutes.

3. Wash in water.

4. Cover with 5 per cent chromic acid  $\frac{1}{2}$  to 2 minutes.

5. Wash in water. Invert and float coverslip on carbolfuchsin solution in a small porcelain dish and heat gently with a flame until it steams; continue this for 3 to 5 minutes. (This step can also be done by covering the coverglass with carbolfuchsin and holding over flame.)

6. Decolorize with 5 per cent sulphuric acid 5 to 10 seconds.

7. Wash in water.

8. Stain with aqueous methylene blue  $\frac{1}{2}$  to 1 minute. By this method spores will be stained red, the body blue.

**Gram's Stain for Capsules.**—Thin smears stained by the gram method as described are frequently satisfactory for capsules.

**Welch's Method for Capsules.**—1. Cover film with glacial acetic acid for few seconds.

2. Drain and replace with aniline gentian violet. Drain and again replace with aniline gentian violet. Repeat until all acid has been replaced by gentian violet solution.

3. Wash in a 1 or 2 per cent solution of sodium chloride and mount in same. Do not use water at any stage.

4. The capsule stains pale violet.

**Hiss's Copper Sulphate Method for Capsules.**—1. Grow organisms in ascitic fluid or serum medium or mix with drop of serum and from this mixture prepare smears.

2. Air dry and fix with heat.

3. Cover preparation with 5 per cent water solution of gentian violet and heat for few seconds until steam arises (5 cc. of saturated alcoholic solution of gentian violet to 95 cc. of distilled water).

4. Wash dye off with 20 per cent solution of copper sulfate crystals.

5. Blot (do not wash) and dry thoroughly.

6. By this method permanent preparations are obtained, the capsules appearing with faint blue halos around dark purple cell bodies.

**Huntoon's Method for Capsules.**—1. Mix the organisms with a small drop of nutrose solution on a slide and spread the film; dry in air (do not fix).

#### NUTROSE SOLUTION

Nutrose (sodium caseinate) .....	3 gm.
Water .....	100 cc.

Cook the solution for 1 hour in Arnold sterilizer and add 0.5 per cent carbolic acid. Place in tubes without filtering.

2. Cover with stain for 30 seconds.

#### STAIN

Carbolic acid (2 per cent aq. sol.) .....	100.0 cc.
Acetic acid (1 per cent aq. sol.) .....	1.0 cc.
Lactic acid (conc.) .....	0.5 cc.
Carbolfuchsin .....	1.0 cc.
Basic fuchsin (sat. alc. sol.) .....	1.0 cc.

3. Wash in water and dry.

4. This method is applicable to cultures only; not to exudates.

**India Ink Method for Capsules.**—On a clean slide mix the culture with a loop of India ink. Spread to a thin film as in making blood smears. Allow to dry; fix. Stain for 1 minute with methylene blue or for a few seconds with basic fuchsin. Wash with water, dry and examine. Only the bacterial bodies are stained, the capsules appearing as clear spaces about the bacteria.

**Fontana's Stain for Spirochetes (Tribondeau).**—For staining spirochetes in smear preparations:

#### SOLUTIONS

(1) Fixing solution.

Acetic acid, glacial .....	1 cc.
Formalin .....	20 cc.
Water .....	100 cc.

(2) Mordant.

Tannic acid .....	5 gm.
Carbolic acid .....	1 gm.
Water .....	100 cc.

## (3) Silver solution.

Silver nitrate .....	1 gm.
Water .....	20 cc.

Dissolve the silver nitrate, then add drop by drop a 10 per cent ammonia solution until the precipitate redissolves. The solution at this stage should be slightly opalescent; if clear, a few drops of silver nitrate solution should be added to give a faint turbidity.

1. Prepare a smear and allow to dry; treat with the formalin fixative for 1 to 2 minutes.

2. Wash thoroughly with absolute alcohol; in case of smears from organs, the fat should be removed by the application of ether after the alcohol and a final application of alcohol.

3. Pour on the tannin mordant and heat till steam arises; continue for half a minute.

4. Wash thoroughly in tap water, then in distilled water.

5. Stain with the silver solution for a few seconds in the cold, then pour off and add fresh silver solution and heat till steam rises for a quarter of a minute.

6. The slide, which should now be a distinct brown color, is washed in distilled water, blotted and dried. The spirochetes should, if the method has been properly carried out, be of a blackish or brown color. The film should be mounted in Canada balsam, since when examined without a coverslip the cedar-wood oil discolors the spirochetes.

**India Ink Method for Spirochetes (Burri).**—On one end of a slide mix an equal amount of secretion from a chancre or other material with India ink free of artefacts. Smear as described for making blood smears. Allow to dry and examine. The spirochetes will appear as white spirals on a black background.

**Tunnell Method for Spirochetes and Fusiform Bacilli.**—1. Make a thin smear of the material on a slide. Fix with heat.

2. Cover with carbol crystal violet (sat. alc. sol. crystal violet, 10 cc.; 5 per cent aqueous phenol sol. 90 cc.) for 30 seconds.

3. Wash with water.

4. Cover with Lugol's iodine solution, 30 seconds.

5. Wash with water.

6. Cover with safranin, 30 seconds.

7. Wash with water.

Spirochetes and fusiform bacilli stain purplish black. Large bacterial forms are often granular. Capsules may be demonstrated by this method occasionally.

**Gracian Method for Rickettsiae.**—1. Prepare smears and fix by heating.

2. Cover with xylol for 3 minutes.

3. Pour off xylol and flood twice with 96 per cent alcohol.

4. Wash with tap water.

5. Cover with a saturated solution of  $K_2Cr_2O_7$  for 3 minutes.

6. Wash with water and stain for 10 to 20 minutes with a 10 per cent Giemsa solution (2 drops to 1 cc. of distilled water).

7. Wash with water and dry (*Ztsch. f. Hyg. u. Infektionsk.*, 17: 6, 1943).



# METHODS OF STAINING BACTERIA IN TISSUES

**Preparation of Tissue Section.**—1. Examine tissue as soon as possible after removal.

2. Place blocks not larger than  $\frac{1}{2}$  by  $\frac{1}{8}$  inch in Zenker's fluid for 3 to 12 hours.
3. Wash in water for several hours.
4. Place for 24 hours in each of the following alcohols in succession: 30, 60, 90 per cent and absolute.
5. Place in cedar oil or xylol until translucent.
6. Place in equal parts of cedar oil or xylol and paraffin at 37° C. for 2 hours.
7. Place in paraffin at 52° C. for 2 hours in each of 2 baths.
8. Box and cut sections of 3 to 6 micra.
9. Dry sections in incubator for about 24 hours or over night.
10. Remove the paraffin by placing the slides in xylol and then in absolute alcohol.

Repeat until all paraffin is removed and then place sections in water (no clouding denotes removal of paraffin).

**Goodpasture's Stain.**—1. Place prepared section in the following stain for 10 to 30 minutes:

Alcohol .....	100.00 cc.
Basic fuchsin .....	0.59 gm.
Aniline oil .....	1.00 cc.
Phenol (crystals) .....	1.00 gm.

2. Wash in water. Place in 40 per cent formalin for a few seconds (bright red color fades to a clear rose). Wash in water.

3. Counterstain in saturated water solution of picric acid for 3 to 5 minutes (until section assumes a purplish-yellow color). Wash in water.

4. Differentiate in 95 per cent alcohol (red appears and some is washed out; some picric acid is washed out). Wash in water.

5. Stain in Sterling's gentian violet for 5 or more minutes:

Gentian violet .....	5 gm.
Alcohol (95 per cent) .....	10 cc.

Grind in a mortar and add:

Aniline oil .....	2 cc.
Water (distilled) .....	88 cc.

Let stand for 1 or 2 days and filter.

6. Wash in water and place in Gram's iodine solution for 5 minutes. Blot dry without washing.

7. Place in aniline oil and xylol (equal parts) until no more color comes away. Place in 2 changes of xylol. Mount in Canada balsam.

8. Gram-negative organisms stain red; gram-positive organisms, blue; tissues stain in shades of red to purple.

**Gram-Weigert Method for Paraffin Sections.**—1. Fixation in Zenker's solution is preferred.

2. Stain sections lightly in alum-hematoxylin. Wash in running water.

3. Four per cent aqueous solution eosin soluble in water, 5 minutes to  $\frac{1}{2}$  hour. Wash in water.

4. Aniline methyl violet  $\frac{1}{2}$  to 1 hour. Wash off with water.

5. Lugol's solution, 1 to 2 minutes. Wash off with water.

6. Blot with filter paper and dehydrate and clear in several changes of aniline and xylol, equal parts, or in aniline oil alone. Wash off with xylol. Mount in xylol-colophonium.

**Pappenheimer's Method for Gram-positive and Gram-negative Organisms.—**

1. Zenker fixation preferred; paraffin sections should be 5 microns or less.

2. Stirling's gentian violet for 5 minutes; Gram's iodine for 1 minute. Decolorize to pale violet with aniline oil or aniline oil and xylol.

3. Absolute alcohol, for a few minutes only.

4. Wash with distilled water.

5. Aqueous safranin,  $\frac{1}{2}$  per cent for 30 seconds.

6. Distilled water and blot.

7. Absolute alcohol; few minutes only. Clear in xylol.

**Brown and Brenn Stain for Gram-positive and Gram-negative Organisms.—**

1. Stain in freshly filtered alum-hematoxylin (Harris) for 2 to 5 minutes.

2. Wash in acid alcohol (3 per cent HCl in 95 per cent alcohol) until light pink.

3. Wash in ammonia water (1 cc. of aqua ammonia in 100 cc. water) until blue.

Wash in water.

4. In a small vial mix 5 drops of 5 per cent aqueous solution of sodium bicarbonate (containing also 0.5 per cent phenol as preservative) with about 0.75 cc. of 1 per cent aqueous solution of gentian violet. Immediately pour the mixture onto the slide and stain for 2 minutes. Wash quickly with water.

5. Cover with iodine solution (iodine 1 gm., potassium iodide 2 gm., water 300 cc.) for 1 minute.

6. Wash with water. Blot.

7. Decolorize in 1 part of ether plus 3 parts of acetone, dropping it onto the slide until no more color comes off. Blot.

8. Stain for 5 minutes with rosaniline hydrochloride (0.005 gm. per 100 cc. water). Wash in water. Blot but do not allow the section to dry.

9. Pass through acetone. Decolorize and differentiate by dropping over the section a solution of 0.1 gm. of picric acid in 100 cc. of acetone until the section becomes yellowish-pink. This is the most critical stage of the process and should be carried out by holding the slide over a white plate or dish. Most of the rosaniline should be decolorized from the tissue but the gram-negative bacteria should remain red. Pass successively through acetone, equal parts of acetone and xylol, and xylol. After clearing in xylol mount in balsam. (Beginning with step 5, it is best to work with only one slide at a time.) Cell nuclei should be stained dark reddish-brown; cytoplasm yellowish; gram-positive bacteria deep violet or almost black; gram-negative bacteria bright red. Leukocytes generally stand out plainly with a dusky yellowish cytoplasm. Basophilic granules stain red. Red blood cells may be a yellow or red depending upon the degree of decolorization in picric acid. Cartilage stains pink. Striated muscle and fibrin generally stain yellow but may retain more or less of the red stain.

**Krajean's Method for Gram-Positive and Gram-Negative Organisms in Frozen Sections.**—1. Prepare frozen sections at 7 to 10 microns in the usual manner.

- 2. Stain for 2 minutes in alum-hematoxylin.
- 3. Wash in tap water until blue and destain rapidly in acid alcohol, dipping in and out 5 to 7 times.
- 4. Rinse in tap water and apply copper sulfate-zinc sulfate solution for 3 minutes (copper sulfate, 7 gm. and zinc sulfate, 4 gm., dissolved in 100 cc. of distilled water by the aid of heat).
- 5. Pour off; apply brilliant green solution for 5 minutes (dissolve 0.3 gm. of hematoxylin crystals in 10 cc. of formalin; add 0.3 gm. of brilliant green; mix thoroughly; filter if a precipitate forms).
- 6. Rinse in tap water and apply carbolfuchsin (Ziehl-Neelsen method) for 2 minutes.
- 7. Rinse in tap water, blot, and apply dioxane for 2 minutes.
- 8. Pour off and without washing apply creosote-xylene (equal parts), changing the solution several times and agitating the slide for even differentiation until the background appears to be clear red with no more stain leaving the section. (This step require about 1 minute, and it is advisable to control the differentiation under the microscope.)
- 9. Clear in pure xylene 2 minutes.
- 10. Mount in gum damar.

With the use of this method, nuclei are bluish red, gram-positive organisms bluish green, gram-negative organisms red, monilias and actinomycetes green, and Negri bodies bright red with greenish chromatin bodies.

**Mallory and Wright's Method for Tubercle Bacilli.—Paraffin Sections.**—1. Stain in carbolfuchsin solution hot for 5 minutes (or better, cold, for 24 hours). Wash in water. Decolorize and counterstain in Gabbet's methylene blue-sulphuric acid mixture for 1 minute:

Methylene blue .....	2 gms.
Sulphuric acid .....	25 cc.
Water .....	75 cc.

- 2. Wash in water. Dehydrate in absolute alcohol. Clear in xylol. Mount in balsam.

**Celloidin Sections.**—Stain lightly in alum hematoxylin. Wash in water. Dehydrate in 95 per cent alcohol. Attach the slide by ether vapor. Stain with steaming carbolfuchsin 2 to 5 minutes. Wash with water. Wash with Orth's acid alcohol (alcohol 90 per cent 99 cc.; conc. hydrochloric acid, 1 cc.) ½ to 1 minute. Wash in water several changes. Treat with 95 per cent alcohol until red color is entirely gone. Blot and cover with xylol until clear. Mount in balsam.

**Second Method for Staining Tubercle Bacilli in Tissue.**—Fix tissue in formol solution or other fixative. After sections have been cut and the paraffin removed, pass them down to water. Stain in carbolfuchsin 3 hours at room temperature.

STOCK SOLUTION

Basic fuchsin .....	10 gms.
Absolute alcohol .....	100 cc.

Use for staining:

Stock solution .....	10 cc.
Phenol 5 per cent .....	90 cc.

Treat slides with acid alcohol (alcohol 80 per cent 98 cc.; hydrochloric acid, 2 cc.) until almost decolorized. Wash in distilled water. Blot. Wash in water. Stain in Harris's hematoxylin, 1 minute. Wash in water. Acid alcohol, 2 to 4 seconds. Wash in water. Blot. Wash in aniline oil. Treat with  $\frac{1}{3}$  aniline oil and  $\frac{2}{3}$  xylol. Clear in xylol and mount in balsam. *Balsam must be neutral or the slides will fade in time.*

**Mallory's Method for Actinomyces.**—Stain deeply in saturated aqueous eosin 10 minutes. Wash in water. Aniline gentian violet from 2 to 5 minutes. Wash in normal saline solution. Weigert's iodine solution (iodine, 1 gm.; potassium iodide, 2 gm.; water, 100 cc.) 1 minute. Wash in water and blot. Clear in aniline oil. Xylol several changes. Mount in balsam.

## METHODS FOR STUDYING BIOLOGICAL PROPERTIES OF BACTERIA

**Study of Colonies.**—*Surface colonies* on agar media are often of considerable value in identification. Such colonies of different bacteria may vary considerably and an experienced bacteriologist can often make a tentative diagnosis by the study of the surface colonies. This is especially true where the bacteria are planted on special media and exhibit peculiar colonial forms. The features of colonies which should be noted especially are whether the colonies are: dry or moist, mucoid, rough or smooth, wrinkled, translucent or opaque, shape of edges, flat or elevated, spreading, and color. By means of a hand lens or the low power of the microscope more details are brought out.

*Deep colonies* usually are less characteristic than surface colonies, except in special media. In blood agar the deep colonies are especially informative and are the basis for the differentiation of the streptococci. Deep colonies are best studied with the lower power of the microscope, or better, by unscrewing the lower half of the low power lens of the microscope a magnification is obtained which seems just right.

**Appearance in Broth.**—The growth in broth is often of diagnostic value. The growth may be homogeneous, granular, flocculent, or mucoid. A pellicle may form on the surface, and a precipitate on the bottom.

**Production of Pigment.**—Many bacteria produce very intense pigments which are characteristic and of considerable value in diagnosis. Such pigments are produced more abundantly in some media than in others. Löffler slants are very good for demonstrating pigmentation. Pigment production is generally best under aerobic conditions and may fail altogether in the absence of oxygen, as in the case of *B. pyocyaneus* and *Serratia marcescens*. In many instances more pigment is produced at lower temperatures so that it is advisable to incubate at temperatures of about 20° C. as well as at 37° C.

**Liquefaction of Gelatin and Coagulated Serum.**—Proteolytic activity of bacteria is usually studied by inoculation into gelatin or on coagulated serum slants. Gelatin liquefaction may be studied by incubating the inoculated gelatin at 37° C. for one or more days and then placing it in cold water to see if it will solidify. A better method is to stab the solid gelatin medium and incubate at 20 to 22° C. By this means the type of liquefaction may be observed and more information is thus obtained.

**Reactions in Milk.**—Several reactions may occur:

- (a) Production of acid by the fermentation of lactose.
- (b) Production of acid and coagulation by sufficient acid to coagulate the casein.
- (c) Production of alkali by the breakdown of nitrogenous compounds into amines, ammonia, etc.
- (d) Peptonization by the breakdown of casein.

Acid production in bromthymol blue milk is shown by a yellow color. If alkali is produced the color becomes deep blue. Peptonization can be detected by the separation of the milk into a thin supernatant fluid (whey) and an undigested sediment. The indicator is frequently destroyed by proteolytic bacteria.

**Action on Carbohydrates.**—A large variety of carbohydrates are used in the study of bacteria. Many of these are unstable and cannot be sterilized in the medium in the autoclave without undergoing some hydrolysis. Such carbohydrates can often be sterilized unchanged in 10 to 20 per cent solution in distilled water and then added aseptically to the medium. Such concentrated sugar solutions are best kept after sterilization in tubes under a vaselin seal (the vaselin being sterilized in the oven at 160° C. for 2 hours before being added to sugar solution), or in tubes with a rubber cap to prevent evaporation of the water. Very delicate carbohydrates must be sterilized by filtration through a sterile bacteriological filter. The carbohydrates are generally added to media in 0.5 per cent or 1 per cent concentration.

In the study of streptococci and allied bacteria, when the final acidity attained is of diagnostic importance, it is best not to add the indicator to the medium. At the end of the incubation period (5 to 7 days) a small amount of the culture is removed and the pH determined by the method of Brown or other satisfactory methods. Where it is only necessary to determine whether acid is produced or not, the indicator may be added to the medium before sterilization.

To demonstrate gas production shake or stab cultures in agar media are often used, or liquid media covered with a petrolatum seal. The Smith type of fermentation tube may be used but by far the most common is the Dunham tube. A small glass tube is inverted in the tube of medium. When the medium is autoclaved the inverted tube becomes filled with the medium. Any gas produced is trapped in the inverted tube.

The amount of gas produced may vary with the conditions of the culture and the nature of the medium. Gas production may be completely inhibited by nitrates or nitrites in as low as 0.1 per cent concentration. The gas produced is usually mainly carbon dioxide and hydrogen. The ratio of the carbon dioxide and hydrogen which is produced is of diagnostic value in differentiating such groups of bacteria as aerobacter and escherichia. The analysis of the gas may be done in a Smith fermentation tube by absorbing the carbon dioxide with sodium hydroxide. The residual gas is mostly hydrogen. Some years ago this test was commonly used but now it has been largely abandoned.

**Action on Organic Acids.**—Some organic acids are used for differentiation of bacteria. Most of these are used in synthetic media as the sole energy source. Among acids thus used may be mentioned citric, malonic and tartaric.

**Production of Indol.**—This seems to be a fundamental and important property of bacteria. Indol is produced from tryptophane and any medium used for this test must contain tryptophane in appreciable quantities. The following tests are recommended:

**Oxalic Acid Paper Test for Indol (Holman and Gonzales).**—Soak filter paper in saturated oxalic acid solution. Dry and cut into strips. Hang a strip of the paper in the form of a loop over the medium in a culture tube, securing the ends of the paper between the cotton plug and the mouth of the tube. Indol is shown by the development of a pink color on the paper during the growth of the culture. The paper must not be allowed to become wet.

**Ehrlich Indol Test:**

REAGENT

Paradimethylaminobenzaldehyde .....	2 gms.
Ethyl alcohol (95 per cent).....	190 cc.
Hydrochloric acid (conc.).....	40 cc.

Add about 1 cc. of ether to tryptone broth culture and shake. Allow ether to rise to the top and form a layer. Add about  $\frac{1}{2}$  cc. of reagent so that it forms a layer between the medium and the ether. A red color forms at the junction and spreads into the ether layer.

**Reduction of Nitrates.**—Reduction of nitrates to nitrites demonstrates the reducing ability of bacteria. This reaction is not of very great diagnostic value except in isolated instances. The reaction may be demonstrated in either agar or fluid media.

**Test for Nitrites:**

SOLUTION A

Sulphanilic acid .....	4 gms.
N/5 acetic acid (Sp. gr. 1.041) .....	500 cc.

SOLUTION B

Alpha-naphthylamine acetate .....	2.5 gms.
N/5 acetic acid (Sp. gr. 1.041).....	500 cc.

Filter through washed absorbent cotton.

To 10 cc. of culture add 0.2 cc. of Solution A. Add Solution B drop by drop until a red color appears. In the presence of nitrites a red azo-compound is formed. The reagents must be added in the order given.

**Cholera Red Reaction.**—For the identification of *V. cholera*. Culture in peptone water for 2 or 3 days. Add a few drops of concentrated sulphuric acid. A positive reaction is indicated by the development of purplish pink color (a nitro-indole reaction).

**Acetyl-Methyl-Carbinol Test (Voges-Proskauer Reaction).**—This test is extensively used for the differentiation of aerobacter and escherichia. The culture in dextrose peptone broth may be tested after 2 to 4 days of incubation. Reagent: Dissolve 1 gm. of copper sulfate in 10 cc. of distilled water and when dissolved mix with 40 cc. of concentrated ammonia water. Add 950 cc. of 10 per cent aqueous solution of sodium hydroxide. Add an equal volume of the reagent to the culture and shake thoroughly. A positive reaction is indicated by the development in 10 to 20 minutes of a red color (not to be confused with the violet tint of the biuret reaction which also occurs).

**Methyl Red Test.**—For differentiating aerobacter and escherichia. The bacteria are cultivated for several (4) days in the special (V-P-M-R) medium. A test for degree of acidity is made with methyl red indicator by adding a few drops of a 0.04 per cent

solution of methyl red in 60 per cent alcohol. A red color (acid) is called a positive test; a yellow color (alkaline) is a negative test.

**Production of Hydrogen Sulfide.**—The production of hydrogen sulfide is of some value in differentiating bacteria but the test must be used with caution: Media for this test must be carefully standardized to get consistent results. By adding cystine or sodium thiosulphate to a medium almost any type of bacteria will produce hydrogen sulfide. The concentration of agar has also a considerable effect. Addition of meat infusion increases hydrogen sulfide production. The usual test for hydrogen sulfide is by means of lead acetate or ferric citrate, which are incorporated into agar media. Blood may also be used and it turns a strong green color in the presence of hydrogen sulfide and oxygen.

Kligler's iron agar (page 357) is one of the most satisfactory media for the detection of hydrogen sulfide. If hydrogen sulfide, a volatile gas, is produced in culture it combines with the colorless iron salt in the medium to form black iron sulfide.

**Analysis of Gas Formed by Bacteria.**—*Carbon Dioxide.*—1. For the estimation both qualitatively and roughly quantitatively of carbon dioxide produced by bacteria, cultures are grown in fermentation tubes containing sugar-free broth to which 1 per cent of pure dextrose, lactose, saccharose, or other sugars has been added.

2. The tubes are incubated until the column of gas formed in the closed arm no longer increases (24 to 48 hours). The level of the fluid in the closed arm is then accurately marked and the column of gas measured.

3. The bulb of the fermentation tube is then completely filled with N/5 sodium hydroxide solution, the mouth closed with a clean rubber stopper, and the bulb inverted several times in order to mix the gas with the alkali. The tube is then again placed in the upright position, allowing the gas remaining to collect in the closed arm. The gas lost may be roughly estimated and considered as being carbon dioxide.

*Hydrogen.*—The gas remaining, after removal of the carbon dioxide in the preceding may be estimated as hydrogen. When allowed to collect near the mouth, further evidence of its being hydrogen may be gained by exploding it with a lighted match.

**Ferric Chloride Test for Hydrolysis of Sodium Hippurate.**—*Reagent:* 12 gms. of ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) dissolved in 100 cc. of 2 per cent hydrochloric acid in water. Transfer 0.8 cc. of culture in sodium hippurate broth to a small test tube (Wassermann tube) and add 0.2 cc. of the reagent. Mix immediately and observe after 10 to 15 minutes. A permanent precipitate indicates the presence of benzoic acid (positive hydrolysis.)

Since sodium hippurate is first precipitated and later redissolved by the amount of reagent specified and since benzoic acid is also redissolved by a greater excess of the reagent, it is necessary to have the reagent and the medium balanced and to measure the amounts used in the test quite accurately. A control test of the sterile medium should always be made. If the culture is quite turbid so as to confuse the reading of the result, it should be centrifuged and the clear supernatant used for the test.

**Solubility Test for Pneumococci with Sodium Desoxycholate.**—*Reagent:*

Sodium desoxycholate .....	10 gms.
Alcohol .....	10 cc.
Water. ....	90 cc.

**Method.**—To 1 cc. of broth culture add 2 drops of reagent. Dissolution of pneumococci generally occurs in less than 5 minutes. Bile is commonly used for this test but the action is much slower and the results are much less clear-cut than with sodium desoxycholate.

**Hemolysin Test.**—For differentiation of human and bovine types of hemolytic streptococci.

With sterile capillary pipet transfer to small serological test tubes 10 to 15 drops of the broth cultures. To each tube add an equal volume of 5 per cent suspension in salt solution of washed rabbit blood cells. Incubate at 37° C. for 2 hours. The amount of laking of the blood cells is indicated by negative, 1 plus, 2 plus, 3 plus and 4 plus.

**Action on Blood Agar.**—Bacterial colonies (deep) in blood agar are of at least 3 types. The designation alpha ( $\alpha$ ), beta ( $\beta$ ) and gamma ( $\gamma$ ) of Smith and Brown has gained general acceptance. Any types of bacteria may produce these appearances but the cause may be different. Gram-negative bacilli producing the alpha type of colony do so because of the hydrogen sulfide, which is produced, while the alpha type of streptococci, or pneumococci, produces no hydrogen sulfide but changes the hemoglobin to hematin which is oxidized to a greenish or brown substance. The alpha type of colony in the case of the cocci is characterized by an immediate zone of unhemolyzed blood cells, usually colored either green or brown. Outside of this zone is a partially clear area. The beta type of colony is the frankly hemolytic type. Recently various bacteria have been found to produce double or even triple zones of hemolysis, especially following refrigeration of the blood plates. Differentiation of the various types of colonies should always be done by means of the low power of the microscope after incubation for 1 or 2 days followed by refrigeration for 1 day. The blood agar plate constitutes one of the most serviceable of the culture media; the size and shape of the colonies frequently lend much aid in the identification of many pathogenic species as briefly summarized in tables 14, 15 and 16.



TABLE 14.—GRAM POSITIVE COCCI WHICH GROW ON BLOOD AGAR

TYPE OF COLONY	MORPHOLOGY	MEDIA FOR SUBCULTURING	IDENTIFICATION METHODS		TENTATIVE CLASSIFICATION
			Pigment	None	
Large, opaque, circular, smooth edges.	Grouped irregularly. Not encapsulated.	Plain agar	Orange		<i>Staphylococcus albus</i>
			Lemon		<i>Staphylococcus aureus</i>
Glistening, circular, smooth edges, viscid.	Grouped in tetrads encapsulated.	Plain agar			<i>Staphylococcus citreus</i>
					<i>M. tetragenus.</i>
Moist, often viscid, smooth edges 1 mm. or more in diameter, green zone surrounds colony.	Occurs in pairs, sometimes chains. Often lancet shaped, capsules usually demonstrable.	Blood infusion broth or Rosenow's brain broth, and Hiss serum water + inulin.		Soluble in bile. Ferments inulin.	Pneumococcus.
Small, green zone surrounds colony.	Occurs in pairs and chains. No capsules.	Blood infusion broth or Rosenow's brain broth, and Hiss serum water + inulin.		Not soluble in bile. Does not ferment inulin.	Streptococcus alpha type. (viridans)
Small, surrounded by a hazy, colorless zone.	Occurs in pairs and chains. No capsules.	as above		as above	Streptococcus alpha prime type.
Small, surrounded by a clear, colorless zone.	Occurs in pairs and chains. No capsules.	as above		as above	Streptococcus beta type. (hemolyticus)
Vary greatly in size. No change in the media surrounding colony.	Occurs in pairs and chains. No capsules.	as above		as above	Streptococcus gamma type. (indifferens)

TABLE 15. —GRAM POSITIVE RODS WHICH GROW ON BLOOD AGAR

TYPE OF COLONY	MORPHOLOGY	SPORES	MOTILITY	IDENTIFICATION METHODS	TENTATIVE CLASSIFICATION
Large with irregular margins.	Straight, slender, sometimes chains.	Spores formed	Non-motile	Pathogenic for mice	<i>B. anthracis</i>
			Non-motile	Not pathogenic for mice	Saprophytic organism
			Motile		Saprophytic organism
Small	Pleomorphic; often contain metachromatic granules.	Not formed	Non-motile	Toxic for Guinea pigs	<i>C. diphtheriae</i>
				Not toxic for Guinea pigs	Diphtheroids

TABLE 16.—GRAM NEGATIVE RODS WHICH GROW ON BLOOD AGAR PLATES

TYPE OF COLONY	MORPHOLOGY	MEDIA FOR SUBCULTURING COLONY	TENTATIVE CLASSIFICATION
Large, opaque; smooth or irregular edges; smooth or rough surface	Short, plump (sometimes encapsulated)	Plain agar	Coli-typhoid-paratyphoid dysentery
Large, opaque, viscid, smooth edges	Short, plump, encapsulated	Plain agar	Klebsella group
Large, opaque, spreading, dirty, discoloration of media, disagreeable odor	Short, plump	Plain agar	No pigment: <i>B. proteus</i> Green Pigment: <i>B. pyocyaneus</i>
Very small, moist	Very tiny	Chocolate agar	Hemophilus group
Small, yellow, round, opaque	Small, stains irregularly	Glycerine—veal agar	<i>B. mallei</i>
Small, translucent, irregular borders	Ovoid, bi-polar, pleomorphic	Plain agar	Pasteurella group
Small, round, regular borders	Small, oval	Plain or blood agar	<i>Br. melitensis</i>

# DIAGNOSTIC BACTERIOLOGICAL METHODS

## REGIONAL DISTRIBUTION OF PATHOGENIC ORGANISMS

1. When material is submitted for bacteriological examination its source should be stated, as this information may indicate the probable bacteriological findings and guide the method of examination; for example, pus from an acute furuncle or "boil" usually shows a staphylococcus in pure culture. It is therefore helpful in bacteriological diagnosis to keep in mind the regional distribution of the pathogenic organisms, although in smears and cultures of the skin and mucous membranes open to air contamination, various nonpathogenic bacteria may be encountered which are not included herewith except such common ones as *B. subtilis*, *B. proteus-vulgaris*, diphtheroid bacilli and such organisms.

2. No attempt has been made to include all the organisms found and described in the different locations listed. For example, at least 46 different ones have been found in the saliva and even a large number in the feces, but the majority of them are of little or no importance. For the identification of these the worker is referred to Bergéy's *Manual of Determinative Bacteriology*.

3. When material like feces containing many different bacteria is submitted for bacteriological examination, the specimen should be accompanied by a request designating the particular organism or organisms to be examined for.

4. Since a smear of material or its culture stained by the Gram method at once yields very useful information, it has proved helpful to arrange the regional distribution of organisms on the basis of this differential stain.

5. The new nomenclature of the American Society of Bacteriologists is given as well as the older names for the various micro-organisms for the assistance this may give in gaining familiarity with the newer terminology.

6. A few of the pathogenic micro-organisms may be identified by stained smears alone (gonococci, diphtheria bacilli, tubercle bacilli, etc.) but the majority require a study of cultures and of their various biological characteristics described on succeeding pages.

## BACTERIOLOGICAL EXAMINATION OF PUS FROM FURUNCLES AND CARBUNCLES

**Principles.**—1. Regardless of location (skin, eyelids, external auditory canal, etc.) *furuncles* (abscesses or "boils") are usually due to infection by *Staphylococcus aureus* or *albus*; "stitch" abscesses and pimples are usually caused by the latter.

2. *Carbuncles* are larger and more severe and are produced by *Streptococcus hemolyticus*, *Staphylococcus aureus* or a mixture of the two.

3. Chronic discharging furuncles and carbuncles may show additional organisms of secondary infection or contamination listed below.

4. *Anthrax* of the skin usually occurs as a severe furuncle with lymphadenitis called the "malignant pustule" caused by *B. anthracis*.

5. Furuncles of the skin may occur in infections by pathogenic fungi as in actinomycosis, blastomycosis and sporotrichosis.

**Method.**—1. Prepare thin smears of pus or serous fluid on slides and stain by the method of Gram:

Gram-positive	{	Staphylococci
	{	Streptococci
	{	<i>B. anthracis</i>
	{	<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
Gram-negative	{	<i>B. subtilis</i>
	{	<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
	{	<i>B. coli</i> ( <i>Escherichia coli</i> )
	{	<i>B. proteus-vulgaris</i>

2. Inoculate a tube of glucose hormone broth and a blood agar slant. If mixed infection is suspected prepare a blood agar plate by surface streak method.

3. Incubate 24 to 48 hours. Examine colonies and prepare smears; stain by Gram method. Employ special methods described for identification of organisms.

### BACTERIOLOGICAL EXAMINATION OF INFECTED WOUNDS

**Principles.**—1. Traumatic wounds are likely to become infected with both aerobic and anaerobic organisms from the skin, clothing, foreign bodies, etc. There is usually a latent period of 12 to 24 hours during which but few bacteria are found.

2. Gangrene is usually caused by one or more of the pathogenic anaerobic bacilli in association with aerobic organisms and is separately described on page 399.

3. In the ordinary infected wound aerobic methods of examination are usually sufficient. If, however, gangrene or tetanus infection is suspected anaerobic culture methods should be included as described under Bacteriological Examination of Gangrene. The areas at which smears and cultures are made should always be those in which bacteria are most likely to be present in large numbers as around foreign bodies, necrotic bone and deep in the sinuses and crevices of the wound.

**Method.**—1. Pus or secretions may be collected on sterile swabs and sent to the laboratory or smears and cultures may be directly prepared.

2. Prepare thin smears on slides and stain by Gram; any of the following organisms may be present:

Gram-positive	{	<i>Staphylococcus aureus</i> and <i>albus</i>
	{	Hemolytic and non-hemolytic streptococci
	{	Pneumococcus ( <i>Diplococcus pneumoniae</i> )
	{	<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
	{	<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
	{	<i>B. subtilis</i>
Gram-negative	{	<i>Spirochaeta vincentii</i>
	{	<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
	{	<i>B. coli</i> ( <i>Escherichia coli</i> )
	{	<i>B. proteus-vulgaris</i> (especially in gunshot wounds)
	{	<i>B. fusiformis</i> ( <i>Fusiformis dentium</i> )

3. Prepare cultures on blood agar plates (surface streak method) and in glucose hormone broth.

4. Incubate 24 to 48 hours. Prepare smears of the broth culture and of different

colonies; stain by method of Gram. Employ special methods for identification of organisms.

**Bacteriological Control of Treatment of Infected Wounds.**—1. There should be no disinfectant treatment of the wound for at least 2 hours before smears and cultures are made.

2. Every 2 or 3 days, smears should be made on slides with a sterile inoculating wire or sterile swabs, care being taken to obtain material from the worst parts of the wound.

3. Dry in the air. Fix with heat. Stain with methylene blue or by Gram's method.

4. Wash with water, dry and examine (with oil-immersion lens and No. 10 ocular).

5. Estimate the average number of bacteria per field. The results may be charted.

6. As long as there are over 50 bacteria per field, closure of the wound is contra-indicated.

7. If 50 or less, make the examination each day and likewise cultures on blood agar for hemolytic streptococci.

8. If smears show gram-positive bacilli resembling the anaerobes, make anaerobic cultures.

9. The presence of hemolytic streptococci in any number contraindicates closure.

10. The presence of a few saprophytes (2 or 3 per field) does not contraindicate secondary suturing of the wound.

11. Cultures made from wounds recently treated with a chlorine compound may be freed from the antiseptic chlorine by obtaining the material on a sterile swab, immersing this in a tube of sterile N/10 sodium thiosulphate solution, and then preparing cultures from this neutralized mixture.

## BACTERIOLOGICAL EXAMINATIONS IN GANGRENE

**Principles.**—1. Gangrene of wounds is usually due to infection with one or more of the pathogenic, anaerobic, spore forming bacilli.

2. As a general rule secondary infection with staphylococci, streptococci, or any of the aerobic organisms mentioned under the Bacteriological Examination of Infected Wounds are usually present.

3. Gangrene due to vascular occlusions as in Buerger's disease, diabetes mellitus, etc., usually shows secondary infection or contamination with aerobic organisms.

4. Gangrene of infected wounds may be predominantly gaseous or phlegmonous. The large, gram-positive, spore forming and anaerobic bacilli responsible for the infection of wounds are usually the result of contamination, as their natural habitat is the intestinal tract of man and animals.

5. Any one or more of the following gram-positive bacilli may be found in smears and cultures:

- (a) *B. welchii* (*Clostridium welchii*) or *B. perfringens* primarily responsible for "gas" gangrene
- (b) *B. oedematis-maligni* (*Clostridium septicum*) primarily responsible for "phlegmonous" gangrene
- (c) *B. oedematiens* (*Cl. oedematiens*) in "phlegmonous" gangrene

- (d) *B. histolyticus* (*Cl. histolyticum*),
- (e) *B. fallax* (*Cl. fallax*)
- (f) *B. sporogenes* (*Cl. sporogenes*); nonpathogenic
- (g) *Cl. Novyi*
- (h) *Cl. sordelli*

6. These include the principal members of the group, but other doubtfully or non-pathogenic species may be present. The identification and differentiation of the bacilli of this group are ordinarily quite difficult although the 2 principal members, *B. welchii* and *B. oedematis-maligni*, are readily identified.

7. Wounds, including gangrene, may be also infected with *B. tetani* and it is good practice to include examination for this bacillus as described below.

**Method.**—1. It is advisable to collect some of the serous or serogaseous exudate or pus for the preparation of smears and cultures; the material may be collected on sterile swabs. Excised portions of necrotic tissue and spicules of bone are also suitable.

2. Prepare smears on slides and stain by the method of Gram (decolorize with alcohol). The above mentioned organisms occur as large, Gram-positive bacilli with or without capsules; spores are not present, but occur in cultures. Staphylococci, streptococci and other Gram-positive and negative organisms mentioned above under Bacteriological Examination of Infected Wounds may be present.

3. Heavily inoculate tubes of Brewer's sodium thioglycollate broth. These are subsequently used only if the primary plating fails.

4. Introduce swabs or fragments of tissue into 10 cc. tubes of Brewer's sodium thioglycollate broth, mix well, and prepare 1:10, 1:100 and 1:1000 dilutions with the broth medium.

5. From each dilution prepare surface plates on blood agar and B-B-L anaerobic agar. Incubate in an anaerobic jar. It frequently aids colony selection if a few plates are also incubated aerobically. The greatest difficulty in plating arises when spreading types are present and especially *Cl. tetani*, *Cl. sporogenes*, *Cl. septicum* and species of *Proteus*. This tendency is considerably reduced if the surface of the medium is kept relatively dry by placing in the jar 1 or 2 Petri dishes half filled with granular calcium chloride.

6. Surface and subsurface colonies may be finished in 18 to 24 hours and usually reach their maximum growth in 24 to 72 hours.

7. Examine the surface and deep colonies for size and shape. The surface colonies may be classified according to Reed and Orr (*Am. Jour. Med. Sci.*, 206: 379, 1943) as follows:

- Form A = large (2 to 4 mm.), raised, smooth to slightly folded with entire or undulate margins.
- Form B = smaller (1 to 2 mm.), raised, smooth to slightly folded or irregular, with entire to undulate or serrate margins.
- Form C = Minute (0.1 to 0.2 mm.), raised, smooth and entire to slightly irregular with short rhizoids.
- Form D = Moderate to large (1 to 5 mm.), raised, irregular, with widespread rhizoids.
- Form E = Irregular granular with delicate spreading rhizoids to irregular spreading rhizoids without any definite formed colony structure.

8. Note the presence or absence of hemolysis.
9. Prepare and examine smears of selected colonies stained by the method of Gram (decolorize with alcohol).
10. Inoculate tubes of the following media with suspicious colonies: (a) iron milk; (b) carbohydrate broths for Clostridia for acid production (dextrose, maltose, lactose, salicin, sucrose); lead acetate broth for hydrogen sulfide production by Clostridia; gelatin medium for Clostridia; nitrate reduction medium for Clostridia; peptone medium for indol production by Clostridia and milk agar for digestion (prepared of equal parts of B-B-L anaerobic agar and skim milk in plates). Incubate in anaerobic jar for 24 to 72 hours and examine for identification as per the key shown in Table 7.

**Animal Inoculation for *B. Welchii*.**—1. Inject a rabbit intravenously with 1 to 3 cc. of a saline suspension of infected material.

2. Five minutes later kill the animal and place the body in an incubator at 37° C. for 12 to 18 hours.

3. *B. welchii* produces distention with gas and bubbles may be formed in heart, arteries and liver. Prepare smears and stain by Gram. Prepare shake cultures in molten glucose agar cooled to 42° C. and incubate for 24 to 48 hours.

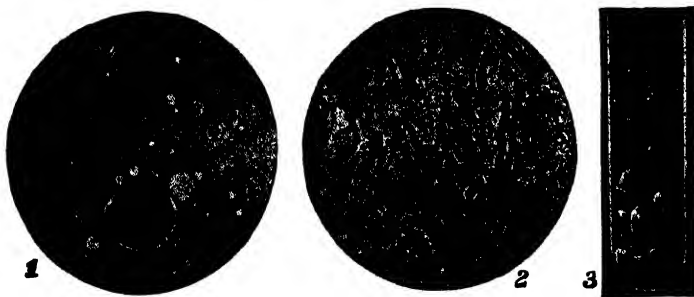


FIG. 165.—*CLOSTRIDIUM WELCHII*

1, Smear from wound. 2, smear from culture. 3, culture tube showing gas formation in agar. (From Park, Williams and Krumwiede, *Pathogenic Microorganisms*, Lea and Febiger, Philadelphia.)

4. Large, gram-positive bacilli which may be encapsulated with “stormy fermentation” of the glucose agar are presumptive evidence of the presence of *B. welchii* (Fig. 165). The method is not infallible, however, as the findings may be due to postmortem invasion by anaerobic organisms from the intestinal tract of the animal.

**Animal Protection Test for Diagnosis of Gaseous and Phlegmonous Gangrene.**—1. Prepare a saline extract of macerated gangrenous tissue or wound secretion.

2. Place 1 cc. into each of 5 small sterile test tubes.

3. Add 1 cc. of the following antisera:

No. 1: Tetanus antitoxin

No. 2: Anti-welchii serum (*Cl. welchii*)

No. 3: Anti-malignant edema serum (*Cl. septicum*)

No. 4: Anti-oedematiens serum (*Cl. oedematiens*)

No. 5: Saline solution (control)



TABLE 17.—KEY FOR THE IDENTIFICATION OF THE CLOSTRIDIA (after Reed and Orr)

Species	Colonies *	Hemolysis	Rods **	Spores ***	Milk ****	Dextrose	Maltose	Lactose	Salicin	Sucrose	H <sub>2</sub> S	Liq. gel.	Reduct. nit.	Indol	Milk agar digestion	Exotoxin
<i>Cl. welchii</i>	A	+ double zone	1	1	S	+	+	+	—	+	+	+	+	—	—	+
<i>Cl. butyricum</i>	A	—	2	2	S	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. multifementans</i>	A	—	2	3	S	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. aerofoetidum</i>	A	—	3	1	S	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. tertium</i>	B	—	4	4	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. fallax</i>	D	—	2	1	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. parapatrificum</i>	B	—	2	4	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. carnis</i>	B	+ single zone	1	4	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. chauvanei</i>	D	+ single zone	8	2	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. septicum</i>	D	+ single zone	8	2	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. sphenoides</i>	B	—	5	4	A	+	+	+	+	+	+	+	+	—	—	—
<i>Cl. novyi</i>	D	+ single zone	8	1	D	+	+	+	—	—	+	+	—	+	+	+
<i>Cl. bifermentans</i>	D	—	1	2	D	+	+	+	—	—	+	+	—	+	+	+
<i>Cl. sordelli</i>	D	+ single zone	1	2	D	+	+	+	—	—	+	+	—	+	+	+
<i>Cl. sporogenes</i>	D	± single zone	6	2	D	+	+	+	—	—	+	+	—	+	+	+
<i>Cl. histolyticum</i>	C	+ single zone	8	1	D	+	+	+	—	—	+	+	—	+	+	+
<i>Cl. tetanomorphum</i>	D	± single zone	4	4	NC	+	+	+	+	+	+	+	—	+	+	+
<i>Cl. difficile</i>	B	+ single zone	2	1	NC	+	+	+	+	+	+	+	—	+	+	+
<i>Cl. capitovialis</i>	B-C	—	4	4	NC	+	+	+	+	+	+	+	—	+	+	+
<i>Cl. cochlearium</i>	D	—	7	4	NC	+	+	+	+	+	+	+	—	+	+	+
<i>Cl. tetani</i>	E	+ single zone	4	4	NC	—	—	—	—	—	+	+	—	—	—	+

\* Surface colonies (see text).

\*\* Rods: 1 = short, thick; 2 = short to long, thick; 3 = short; 4 = short to long, slender; 5 = short, oval to fusiform; 6 = short to long, filaments; 7 = long, very slender; 8 = short to long.

\*\*\* Spores: 1 = subterminal; 2 = excentric to subterminal; 3 = central to excentric; 4 = terminal.

\*\*\*\* Reduced iron milk: S = stormy; A = acid; D = digested; NC = no change.

4. Place in incubator for 30 minutes.
5. Inject into 5 guinea-pigs (subcutaneously) respectively.
6. The control becomes sick in 6 to 12 hours and usually dies.
7. The one or ones protected by serum show no reactions and indicate the nature of the infection.

### BACTERIOLOGICAL EXAMINATION FOR CLOSTRIDIUM TETANI

**Principles.**—1. So few tetanus bacilli are ordinarily present in wounds producing tetanus that the bacteriological diagnosis is usually quite difficult.

2. Whenever possible material for bacteriological examination like pus, tissue scrapings and foreign substances should be obtained from the depths of the wound. These may be collected on sterile swabs or emulsified in a small amount of sterile saline solution and sent to the laboratory.

**Method.**—1. Prepare smears and stain with carbolfuchsin or the method of Gram. Examine for moderately gram-positive bacilli with terminal spores. This method of examination, however, is of very little practical value.



FIG. 166.—TETANUS BACILLI; SPORE STAIN

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

2. Inoculate the material in (a) cooked meat medium; (b) in glucose hormone broth and (c) on a blood agar plate. Incubate at 37° C. for 72 hours under strict anaerobic conditions. Prepare and stain smears. Look for moderately gram-positive bacilli with terminal spores or drumstick forms (Fig. 166).

3. Pure cultures are rarely found. For isolation heat the broth cultures at 75 to 80° C. for 30 minutes in a water bath to kill nonsporing organisms and inoculate blood agar plates for anaerobic cultivation. Also inoculate the water of condensation of an

agar slant and cultivate anaerobically in an upright position. Tetanus bacilli produce an effuse, tenacious proteus-like growth over the surface of the slant. Subcultures from the edge of this fern-like growth into the water of condensation of a fresh agar slant will usually yield a pure culture after several transfers.

4. Test for motility; *Cl. tetani* are motile.

5. Inoculate iron milk, cooked meat medium, milk plate agar and tubes of dextrose, maltose, sucrose, lactose and salicin broth. Cultivate anaerobically. *Cl. tetani* produces no change in milk; slight gas with no blackening or digestion of the cooked meat medium; no acid with the 5 sugars (Table 17).

6. It is always advisable to use a portion of the material for inoculation of 8 to 10 ounce guinea-pigs. Prepare a finely divided emulsion in 2 or 3 cc. of sterile saline. Inject 1 cc. into the thigh of a pig. Inoculate a second pig with the same amount and also inject 500 units of tetanus antitoxin subcutaneously. In the presence of tetanus bacilli or spores the first animal is apt to develop tetanus and succumb in 1 to 4 days while the second survives with few or no symptoms.

7. In a clinical case of tetanus, toxin may be found in the cerebrospinal fluid. Inject 2 or 3 cc. into the thigh of a young pig. Inject a second animal with the same amount along with 500 units of antitoxin. In the presence of toxin the first animal may develop symptoms or succumb in 1 to 4 days.

## BACTERIOLOGICAL EXAMINATION OF LYMPHATIC GLANDS

**Principles.**—1. The bacteriology of the lymphatic glands is quite varied and their bacteriological examination is greatly aided by a knowledge of the clinical aspects of each case for the selection of technic to be employed.

2. Suppurative lymphadenitis is always likely to occur in association with infected wounds and lymphangitis. When pus is examined bacteriologically immediately after spontaneous discharge or incision, the infecting organism is likely to be found in pure culture and is usually a *streptococcus* or *Staphylococcus aureus*.

3. Suppurative infection of the lymphatic glands of the neck is usually secondary to infection of the throat and the pus is likely to show a *streptococcus*, *Staphylococcus aureus*, a pneumococcus or a mixture of these organisms.

4. Any of the superficial lymphatic glands and especially those of the neck may become infected with *B. tuberculosis* producing tuberculous adenitis. When suppuration occurs infection with staphylococci or streptococci is commonly encountered.

5. Chronic open suppurative and tuberculous adenitis almost always shows mixed secondary infection or contamination with any of the following organisms in addition to those of the primary infection: *B. pyocyaneus*; *B. pseudodiphtheriae*; *B. coli*; *B. proteus-vulgaris*; *B. subtilis*, etc.

6. Suppurative adenitis of the groin in association with gonorrhea is usually due to mixed infection with the gonococcus, *Staphylococcus pyogenes*, or *B. coli*, a streptococcus.

7. In *chancroidal infection* the pus from the associated adenitis usually shows the *B. ducrey* in pure culture or in association with staphylococci and streptococci.

8. In bubonic plague *B. pestis* is the infecting organism.

9. Lymphadenitis is also commonly associated with the ulceroglandular type of *tularemia*. Bacteriological examination of the pus may show the presence of *B.*

*tularensis*, usually in association with a staphylococcus or a streptococcus; sometimes either or both of the latter are found in smears and cultures.

10. The lymphatic glands are usually infected during the early stages of syphilis but rarely suppurate. Examinations for *Spirochæta pallida* are sometimes requested.

11. Suppurative lymphadenitis due to *mycotic infections* as in actinomycosis, sporotrichosis and blastomycosis is likely to show the primary organism in the pus usually in association with a staphylococcus, streptococcus or other organisms of secondary infection.

12. Chronic adenitis in association with Hodgkin's disease, lymphatic leukemia, etc., rarely suppurate and bacteriological examination of these, as well as the enlarged glands of cancer and sarcoma, are apt to reveal the presence of pseudodiphtheria bacilli and other organisms without etiological relationship to the primary disease.

**Method for Examination of Pus in Suppurative Lymphadenitis.**—1. The pus should be secured by incision or aspiration or as soon as possible after spontaneous rupture. It may be collected on sterile gauze or swabs.

2. Prepare thin smears on slides and stain by the method of Gram.

3. Inoculate a tube of glucose hormone broth and blood agar plates.

4. Incubate at 37° C. for 24 to 48 hours. Examine colonies; prepare smears and stain by Gram.

5. In ordinary acute lymphadenitis a *streptococcus* or *Staphylococcus aureus* is usually found. In chronic suppurative lymphadenitis these may be present along with organisms of secondary infection or contamination like *B. pyocyaneus*, *B. pseudodiphtheriae*, *B. proteus-vulgaris*, *B. coli*, *B. subtilis*, etc.

**Method for Examination for Tubercle Bacilli.**—1. Prepare smears on slides and stain by Ziehl-Neelsen method.

2. Examine carefully for acid-fast bacilli.

3. If present they are likely to be tubercle bacilli, but this diagnosis should be confirmed by culture and guinea-pig inoculation.

4. If a large amount of pus is available and direct smears are negative, one of the concentration methods may be employed.

5. Prepare cultures on suitable media.

6. Conduct a guinea-pig inoculation test.

7. If a gland has been removed, place a portion in 4 per cent formalin for microscopic examination of sections. The balance should be finely minced or emulsified under aseptic conditions in sterile saline. Cultures are then prepared and guinea-pigs inoculated.

## BACTERIOLOGICAL EXAMINATION OF FISTULAE

**Principles.**—1. Fistulae are usually chronic with mixed infection or contamination by two or more organisms.

2. They may occur in connection with tuberculous or pyogenic osteomyelitis and arthritis; following operations; in actinomycosis or other fungus infections of the skin; sarcoma and carcinoma, etc.

3. It is sometimes difficult to determine the nature of the primary infection because of the presence of secondary organisms like *B. pyocyaneus*, *B. pseudodiphtheriae*, *B. proteus-vulgaris*, *B. coli*, *B. subtilis*, etc.

4. Whenever possible fragments of tissue or bone or scrapings should be examined rather than free pus. Otherwise material should be obtained from the deeper parts on sterile swabs.

**Method.**—1. Prepare thin smears on slides and stain by the Gram method. If tuberculosis is suspected stain by the Ziehl-Neelsen method and examine carefully for acid-fast bacilli. If actinomycosis, blastomycosis or sporotrichosis are suspected examine wet preparations.

2. Prepare cultures on blood agar plates for the ordinary organisms. Bits of tissue or fragments of bone may be placed in glucose hormone broth, incubated at 37° C. for 24 hours, smears examined and plates prepared for isolation if more than one organism is present.

3. If tuberculosis is suspected prepare cultures although it is difficult to secure the tubercle bacilli under these conditions. Conduct a guinea-pig inoculation test.

4. If a pathogenic fungus or yeast is suspected use Sabouraud's medium.

### BACTERIOLOGICAL EXAMINATION OF ULCERS

**Principles.**—1. Ulcers may occur on the skin, cornea and various mucous membranes (conjunctivae, nose, throat, mouth, vagina, urethra, bladder, rectum, etc.) due to a wide variety of infections with pathogenic bacteria, fungi, yeasts, etc.

2. The methods of examination to be employed, therefore, will largely depend upon the nature of the infection suspected.

3. Secondary infection or contamination is common and the bacteriological detection of the primary or specific infection may be difficult.

4. Ulcers due to vascular occlusion or trophic changes invariably become infected or contaminated with pathogenic and nonpathogenic organisms.

5. Material may be collected as described on page 305. The method is important, as mere surface collections may fail to reveal the organism of primary infection. An effort should be made to collect material from the base or depths of the ulcer after cleansing. Sometimes removal of tissue by biopsy is required for acceptable bacteriological examination.

**Method.**—1. Prepare thin smears. Stain by method of Gram. Stain for acid-fast bacilli if tuberculosis is suspected. Examine fresh material by darkfield method if syphilis or Vincent's infection is suspected. Examine wet preparations if infection with fungi or yeasts is suspected. Ordinarily such direct methods are more valuable than cultures for diagnostic purposes.

2. Inoculate glucose hormone broth and prepare blood agar plates by surface streak method for ordinary pyogenic organisms. Use Petraghini medium if infection with *B. tuberculosis* is suspected. Use Sabouraud's medium for fungi and yeasts.

3. Guinea-pig inoculation is best for the detection of *B. tuberculosis*; testicular inoculation of rabbits may be employed in the detection of *Spirochaeta pallida*.

4. Employ special methods for identification of organisms.

### BACTERIOLOGICAL EXAMINATION OF THE EYES

**Principles.**—1. Infections of the eye in relation to bacteriological diagnosis are usually those involving the lids (hordeola and blepharitis); the conjunctivae (acute and chronic conjunctivitis); the cornea and fluid of the anterior chamber (acute and

chronic keratitis with or without ulcers); the iris and anterior chamber fluid (in acute and chronic iritis); the lacrimal ducts and sac (dacrocystitis) and sometimes the lens, choroid, retina and fluid of the posterior chamber.

2. Smears and cultures of the conjunctival sac are also sometimes required before iridectomy and other operations for the purpose of determining whether or not preparatory disinfection is required.

3. Blood agar slants and plates as well as tubes of glucose hormone broth are suitable for the preparation of cultures. When pus and secretions are collected on sterile swabs, smears and cultures should be promptly made.

4. Material may be collected as described on page 306. Well prepared smears of pus or secretions are of great value in the bacteriological diagnosis of conjunctivitis. Local treatment should be stopped until sufficient is obtained for smears and cultures.

**Method.**—1. Stain thin smears by the method of Gram and examine. Additional smears may be stained with methylene blue or diluted carbolfuchsin. Fresh wet preparations should be made and examined in suspected infection with fungi and yeasts.

2. Cultures in glucose hormone broth, on blood agar plates or tubes should be incubated at 37° C. for 24 to 48 hours and examined. Special culture media are required for *B. tuberculosis*, *B. tularensis*, fungi and yeasts, etc.

3. Material to be examined for *B. tuberculosis* may be examined in smears stained by the Ziehl-Neelsen method but guinea-pig inoculation is required.

4. The following organisms may be found in infections of these parts:

Gram-positive	{	<i>Staphylococcus aureus</i> and <i>albus</i>
		Streptococci, both hemolytic and nonhemolytic
		Pneumococci
		<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
		<i>B. xerosis</i> ( <i>Corynebacterium xerosis</i> )
		<i>B. anthracis</i>
		<i>B. subtilis</i>
		<i>Oidium albicans</i>
		Leptothrices
		Streptothrices
		<i>Actinomyces hominis</i>
		<i>Aspergillus fumigatus</i>
Gram-negative	{	<i>Gonococcus</i> ( <i>Neisseria gonorrhoeae</i> )
		<i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
		<i>Meningococcus</i> ( <i>Neisseria intracellularis</i> )
		<i>Bacillus Morax-Axenfeld</i> ( <i>Hemophilus lacunatus</i> )
		<i>Bacillus Koch-Weeks</i> ( <i>Hemophilus conjunctivitis</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		<i>B. tularensis</i> ( <i>Pasteurella tularensis</i> )
		<i>B. Zur-Nedden</i>

### BACTERIOLOGICAL EXAMINATION OF THE NOSE AND ACCESSORY SINUSES

**Principles.**—1. Methods for the collection of material are important and are described on page 307; as a general rule, these should be made by a rhinologist.

2. The bacteriology of these parts is quite varied and special methods are required for certain organisms.

3. In suspected leprosy prepare smears on slides and stain for acid-fast bacilli by the Ziehl-Neelsen method. In leprosy of the skin a nodule may be removed by biopsy. Place a portion in 4 per cent formalin for microscopical examination of sections. Smears may be prepared and stained for *B. leprae*.

4. Furuncles of the atrium are caused by *Staphylococcus aureus* or *albus*.

5. The etiological agent of the acute infectious "cold" or coryza is apparently the filtrable virus of Dochez. Later in the disease secondary infection with pyogenic and other organisms commonly occurs.

**Method.**—1. Prepare smears on slides and stain by method of Gram and methylene blue. Smears for *Spirochaeta vincentii* and *B. fusiformis* should be stained with carbolfuchsin. Smears for tubercle bacilli should be stained by the Ziehl-Neelsen method and carefully examined. In suspected chancre, a darkfield examination for *Spirochaeta pallida* is required.

2. Inoculate tubes of glucose hormone broth and blood agar for pyogenic organisms. Use Löffler's blood serum for *B. diphtheriae*. Use blood agar or Brewer's thioglycollate agar plates if mixed infection is suspected, as in chronic sinusitis.

3. Incubate 24 to 48 hours. Examine smears stained by the Gram method and methylene blue. Any of the following organisms may be present:

Gram-positive	{	<i>Staphylococcus aureus</i> and <i>albus</i>
		Streptococci
		Pneumococci
		<i>B. segmentosus</i> ( <i>Corynebacterium segmentosum</i> )
		<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
Gram-negative	{	<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
		<i>B. subtilis</i>
		<i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
		Meningococcus ( <i>Neisseria intracellularis</i> )
		<i>B. Mucosus ozaenae</i> ( <i>Klebsiella ozaenae</i> )
		<i>B. rhinoscleroma</i> ( <i>Klebsiella rhinoscleromatis</i> )
		Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> )
		<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>B. fusiformis</i> ( <i>Fusiformis dentium</i> )
Acid-fast	{	<i>B. pertussis</i> ( <i>Hemophilus pertussis</i> )
		<i>B. mallei</i> ( <i>Pfeifferella mallei</i> )
		<i>B. proteus vulgaris</i>
		<i>Spirochaeta vincentii</i> ( <i>Borrelia vincentii</i> )
	{	<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )
		<i>B. leprae</i> ( <i>Mycobacterium leprae</i> )

## BACTERIOLOGICAL EXAMINATION OF THE NASOPHARYNX, THROAT AND TONSILS

**Principles.**—1. It is important that smears and cultures be properly prepared; methods are described on page 307.

2. Whenever possible the particular infection suspected or to be examined for should be stated to guide technic, choice of culture medium, etc.

3. Smears on slides or wet preparations for darkfield examination are required for the detection of *Spirochaeta vincentii* and *B. fusiformis*. Smears may be of some value in the detection of diphtheria bacilli, but when negative should never be allowed to exclude the possibility of this infection. With the exception of Vincent's angina and tuberculosis, cultures are required for the bacteriological examination of these parts.

**Methods.**—1. Smears on slides prepared direct or from swabs for examination for Vincent's infection may be stained with dilute carbolfuchsin; otherwise the method of Gram is preferred.

2. Smears for tubercle and leprae bacilli should be stained by the Ziehl-Neelsen method.

3. For diphtheria bacilli use tubes or plates of Löffler's blood serum media. For the meningococcus, sheep serum or other special media in plates may be employed. For other organisms as streptococci, staphylococci, pneumococci and bacilli of the hemophilic group, inoculate plates of blood agar and tubes of glucose hormone broth.

4. Tonsil and adenoid tissue removed at operation are best prepared for culture by grinding the specimen in a sterile mortar together with 3 cc. sterile broth and a small amount of sterile sand until an even suspension is obtained. Streak a blood agar plate with 1 loopful of suspension and inoculate a tube of broth with 0.5 cc. Shake the broth well and pour a deep blood agar plate with 1 loopful. Incubate the plates for 24 hours, and examine for beta hemolytic streptococci and other organisms. Re-incubate for 24 hours and examine for minute beta hemolytic streptococci. Refrigerate overnight and examine for double-zone beta hemolytic streptococci. Prepare smears and stain by the Gram method.

Gram-positive	{	<i>Staphylococcus aureus</i> and <i>albus</i>
		Streptococci
		Pneumococci
		<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
		<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
Gram-negative	{	<i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
		<i>M. flavus</i> ( <i>Diplococcus perflava</i> )
		<i>M. pharyngis siccus</i> ( <i>Diplococcus siccus</i> )
		Meningococcus ( <i>Neisseria intracellularis</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>B. fusiformis</i> ( <i>Fusiformis dentium</i> )
		<i>B. pertussis</i> ( <i>Hemophilus pertussis</i> )
		<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		<i>B. proteus vulgaris</i>
		Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> )
		<i>Spirochaeta vincentii</i> ( <i>Borrelia vincentii</i> )



Acid-fast      { *B. tuberculosis* (*Myobacterium tuberculosis*)  
                       *B. leprae* (*Mycobacterium leprae*)

### BACTERIOLOGY OF THE MOUTH, TEETH AND GINGIVAE

**Principles.**—1. The collection of material for the bacteriological examination of the teeth and gingivae is very important and satisfactory methods are described on page 309.

2. Gingival pus and secretions require the examination of fresh wet preparations for spirochetes and endamebae as well as the examination of stained smears and cultures.

3. The results of darkfield examination of ulcers for *Spirochaeta pallida* must be interpreted with great care and caution because of the chances of error with *Spirochaeta microdentium* and other spirochetes which are commonly found in the saliva.

4. For mycotic infections the examination of fresh wet preparations and stained smears is required in addition to cultures.

**Method.**—1. Prepare smears and stain with diluted carbolfuchsin and by the method of Gram.

2. Prepare and examine wet preparations for *Endamocha gingivalis*, *Oidium albicans*, etc.

3. Darkfield examination of fresh wet preparations may be conducted for the detection of spirochetes.

4. Prepare cultures on plates of blood agar or thioglycollate agar by the surface streak method. The apices of extracted teeth are best cultured in Rosenow's brain broth or glucose hormone broth.

5. After incubation for 24 to 48 hours prepare smears stained by Gram method. For the isolation of mixed cultures inoculate blood agar plates by the surface streak method. Anaerobic cultures are sometimes required.

6. The bacterial flora is extensive and variable; the chief organisms may be listed as follows:

Gram-positive	{	<i>Staphylococcus aureus</i> and <i>albus</i>
		Streptococci
		Pneumococci
		<i>M. tetragenus</i> ( <i>Gaffkya tetragena</i> )
		<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
		<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
		<i>B. acidophilus</i> ( <i>Lactobacillus acidophilus</i> )
		<i>B. mesentericus ruber</i> ( <i>B. teres</i> )
		<i>B. mesentericus-vulgatus</i> ( <i>B. graveolens</i> )
		<i>B. mesentericus-fuscus</i> ( <i>B. mesentericus</i> )
		Various sarcinae
		<i>Leptotrichia buccalis</i>
		Streptothrices
		<i>Oidium albicans</i>

Gram-negative	{	<i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
		<i>B. fusiformis</i> ( <i>Fusiformis dentium</i> )
		<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>Vibrio sputigenus</i>
		<i>B. proteus—vulgaris</i> ( <i>Proteus zenkeri</i> )
		<i>Spirochaeta vincentii</i> ( <i>Borrelia vincentii</i> )
Acid-fast	{	<i>Spirochaeta microdentium</i> ( <i>Treponema microdentium</i> )
		<i>Spirochaeta macrodentium</i> ( <i>Treponema macrodentium</i> )
		<i>Spirochaeta mucosum</i> ( <i>Treponema mucosum</i> )
		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

### BACTERIOLOGICAL EXAMINATION OF THE EARS AND MASTOIDS

**Principles.**—1. It is always advisable to prepare cultures of pus in acute otitis media as soon as possible after paracentesis tympani or after spontaneous rupture, since pure cultures of the infecting organism are thereby usually obtained. In chronic suppurative otitis media, mixed cultures with two or more organisms are usually observed.

2. It is good practice to routinely culture all cases of mastoiditis at the time of operation. Pure cultures are the rule and in case of subsequent complications, like lateral sinus thrombosis with septicemia or meningitis, valuable information will be previously obtained if all cases are routinely cultured beforehand.

3. The technic for obtaining and culturing material of these parts for bacteriological examination is very important and described on page 310.

**Method.**—1. Stain smears by the method of Gram and examine. They are of limited value and cultures are preferred.

2. Cultures are best prepared on slants or plates (preferred) of blood agar or Brewer's thioglycollate agar; plain agar should not be used. Löffler's blood serum is acceptable. At the same time it is advisable to inoculate tubes of glucose hormone broth or Rosenow's brain broth medium as these are best for the cultivation of streptococci and pneumococci.

3. If the presence of fungi is suspected streak two slants of Sabouraud's agar, leaving one at room temperature and the other at 37° C. for 2 weeks before reporting as negative.

4. Incubate 24 to 48 hours. Examine colonies. Prepare and stain smears by Gram method. Streptococci must be differentiated from pneumococci. Identify organisms by proper methods:

Gram-positive	{	<i>Staphylococcus aureus</i> and <i>albus</i>
		Streptococci
		Pneumococci
		<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
		<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )

Gram-negative	<i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
	Meningococcus ( <i>Neisseria intracellularis</i> )
	<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
	<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
	<i>B. fusiformis</i> ( <i>Fusiformis dentium</i> )
	Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> )
	<i>B. proteus vulgaris</i>
	<i>Spirochaeta vincenti</i> ( <i>Borrelia vincenti</i> )
Acid-fast	<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

### BACTERIOLOGY OF THE SPUTUM AND EXUDATES OBTAINED BY BRONCHOSCOPIC ASPIRATION

**Principles.**—1. Methods for the collection of sputum and bronchial secretions for bacteriological examination are given on page 308. They are particularly important in relation to bacteriological diagnosis and the preparation of autogenous vaccines.

2. In the different types of suppurative pneumonitis (lung abscess; bronchiectasis, etc.) anaerobic cultures should be included in addition to aerobic cultures.

3. The bacterial flora ordinarily includes many different bacteria as well as various fungi and yeasts. Whenever possible the particular organism suspected clinically should be specified in order to guide the bacteriological technic.

**Method.**—1. Routinely prepare and carefully examine smears stained by the Ziehl-Neelsen technic for tubercle bacilli. Several specimens of sputum may be required for examination before tubercle bacilli are found.

2. Cultures for tubercle bacilli on special media are advisable.

3. Guinea-pig inoculation for tubercle bacilli may be required.

4. Make a darkfield examination for spirochetes or smears may be stained with diluted carbolfuchsin. Prepare smears and stain by the method of Gram.

5. Prepare and examine fresh wet smears for fungi and yeasts. Prepare cultures on Sabouraud's medium, if infection with these organisms is suspected.

6. Prepare blood agar plates by the surface streak method or inoculate tubes of glucose hormone or Rosenow's brain broth media and plate after 24 hours' incubation. It is advisable to include anaerobic incubation with duplicate cultures.

7. The more important organisms are as follows:

Gram-positive	<i>Staphylococcus aureus</i> and <i>albus</i>
	Streptococci
	Pneumococci
	<i>M. tetragenus</i> ( <i>Gaffkya tetragena</i> )
	<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
	<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
	Blastomycetes
	Actinomyces
	<i>Actinomyces hominis</i>
	<i>Actinomyces asteroides</i>
	Streptothrices
	<i>Leptotrichia buccalis</i>
	<i>Oidium albicans</i>

Gram-negative	{	<i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
		Meningococcus ( <i>Neisseria intracellularis</i> )
		Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> )
		<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		<i>B. fusiformis</i> ( <i>Fusiformis dentium</i> )
Gram-negative	{	<i>B. pertussis</i> ( <i>Hemophilus pertussis</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>B. proteus vulgaris</i>
		<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>Spirochaeta vincenti</i> ( <i>Borrelia vincenti</i> )
		<i>Spirochaeta microdentium</i> ( <i>Treponema microdentium</i> )
Acid-fast	{	<i>Spirochaeta macrodentium</i> ( <i>Treponema macrodentium</i> )
		<i>Spirochaeta mucosum</i> ( <i>Treponema mucosum</i> )
		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

## BACTERIOLOGICAL EXAMINATION OF THE CEREBROSPINAL FLUID

**Principles.**—1. Cerebrospinal fluid should be collected under rigid aseptic precautions to prevent accidental contamination with *Staphylococcus albus* and other organisms of the skin.

2. Bacteriological examinations should be made as soon as possible after collection as otherwise delicate organisms and especially the meningococcus, may not survive.

3. Relatively large amounts of fluid or sediment should be cultured (0.5 to 1 cc.) instead of only 1 or 2 loopful.

4. Smears and cultures are required routinely. Fluids showing no organisms in direct smears may yield positive cultures; or organisms may be found in direct smears of fluid or sediment with sterile cultures.

5. Spinal fluid is normally crystal clear. Opalescence to turbidity usually indicates the presence of pleocytosis due to meningitis in case blood is absent. Perfectly clear fluids, however, may be observed in tuberculous meningitis.

**Method.**—1. The examination of stained smears is very important. These may be prepared of cloudy fluid. It is better to remove a portion with a sterile pipet; centrifuge thoroughly. The supernatant fluid may be used for protein, sugar and other determinations. Prepare smears of the sediment. Stain by the method of Gram and with methylene blue. Definitely gram-negative diplococci (intracellular or extracellular) justifies a provisional diagnosis of meningococcus meningitis. Such smears should be carefully controlled by staining on the same slide, a known gram-positive organism, to prevent decolorized gram-positive cocci being mistaken for meningococci.

2. Beta hemolytic streptococci appear as round gram-positive cocci of uniform size and shape, occurring singly, in pairs, and in short chains. Often they may resemble staphylococci, but it is usually possible to distinguish between them.

3. Pneumococci appear as large, gram-positive, lancet-shaped diplococci. Frequently the capsule is apparent as a clear, narrow halo surrounding the organisms.

4. Staphylococci appear as round, gram-positive cocci occurring singly, in pairs, and in very small, irregular groups of but 3 or 4 organisms.

5. *Hemophilus influenzae* are ordinarily very difficult to detect, usually appearing

as minute gram-negative bacilli obscured by debris and cells. For this reason they are easily overlooked and the smears must be examined very carefully.

6. *Listerella monocytogenes* occur as gram-positive bacilli readily mistaken for diphtheroid bacilli.

7. If the meningococcus is suspected, transfer 1 to 2 cc. of uncentrifuged spinal fluid to a blood agar slant. Heat the tube gently above the level of the medium, cut off the cotton plug even with the mouth of the tube, and immediately insert a sterile rubber stopper. Incubate at 37° C. for 24 to 72 hours. The heating causes the expulsion of some of the air from the tube with a reduction in oxygen tension. Otherwise, streak a "chocolate" agar plate with sediment and incubate 48 hours in a jar carrying about 10 per cent carbon dioxide.

8. If streptococci, pneumococci, staphylococci or *H. influenzae* are suspected, streak the surface of a blood agar plate with sediment and inoculate a tube of infusion broth enriched with a few drops of sterile blood. Also inoculate a tube of sodium thioglycollate agar. Incubate at 37° C. Do not discard cultures as sterile before 4 or 5 days' incubation. Differentiate streptococci from pneumococci by appearance of colonies, bile solubility, inulin fermentation and agglutination tests.

9. In suspected tuberculous meningitis stain smears of sediment by the Ziehl-Neelsen or fluorescent methods. If a coagulum has formed, carefully tease out a portion on a slide, dry and stain. A careful search should be made for tubercle bacilli (usually requires about an hour). Inoculate slants of Petragnini's medium with sediment and incubate at 37° C. Inoculate guinea-pigs with sediment or coagulum.

10. *Spirochaeta pallida* is best detected by intratesticular inoculation of rabbits with 1 to 3 cc. of fluid immediately after removal.

11. Acute *primary* meningitis is usually due to the meningococcus,, streptococcus, pneumococcus or *B. influenzae*.

12. Acute *secondary* meningitis complicating otitis media, mastoiditis, sinusitis and injury is usually due to the streptococcus, pneumococcus or staphylococcus. These and other organisms less commonly encountered are as follows:

Gram-positive	{	Streptococci
		Pneumococci
		Staphylococci
		<i>M. tetragenus</i> ( <i>Gaffkya tetragena</i> )
		<i>Listerella monocytogenes</i>
		Actinomyces
		Yeasts
Gram-negative	{	Meningococcus ( <i>Neisseria intracellularis</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>B. typhosus</i> ( <i>Eberthella typhi</i> )
		<i>B. pestis</i> ( <i>Pasteurella pestis</i> )
Acid-fast		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

## BACTERIOLOGICAL EXAMINATION OF PERITONEAL EXUDATES AND TRANSUDATES

**Principles.**—1. Peritoneal transudates (*ascites*) when collected aseptically are usually sterile, but sometimes contain diphtheroid bacilli or other organisms detected by aerobic or anaerobic cultivation. Fluids are sometimes contaminated with *Staphylococcus albus* and other organisms from the skin.

2. Peritoneal exudates (*pus*) in cases of localized or diffuse peritonitis may show one or more organisms depending upon the cause of peritonitis. In suppurative appendicitis with rupture and in peritonitis following the rupture of peptic ulcers and perforating wounds of the intestines, the infection is always a mixed one with intestinal organisms and anaerobic cultures for *B. welchii* and other anaerobic spore-forming bacilli should be included in the bacteriological examination.

**Method.**—1. Centrifuge and prepare smears of the sediment; stain by the method of Gram.

2. Inoculate blood agar plates with 0.5 to 1 cc.; also inoculate tubes of Rosenow's brain broth or glucose hormone broth. Cultivate one set aerobically for 24 to 48 hours and the second set anaerobically for several days. Examine colonies. Prepare smears and stain by the method of Gram. Identify organisms.

3. If tuberculous peritonitis is suspected stain smears by the Ziehl-Neelsen method and examine very carefully for acid-fast bacilli. Prepare cultures on special media; inoculate guinea-pigs.

4. The bacteriology may be quite varied, but the following embrace the organisms usually encountered:

Gram-positive	{	Staphylococci
		Streptococci
		Pneumococci
		<i>B. welchii</i> ( <i>B. aerogenes-capsulatus</i> )
		<i>B. pseudodiphtheria</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
		<i>B. subtilis</i>
Gram-negative	{	<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		<i>B. proteus vulgaris</i>
		<i>B. typhosus</i> ( <i>Eberthella typhi</i> )
Acid-fast		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

## BACTERIOLOGICAL EXAMINATION OF PLEURAL AND PERICARDIAL EXUDATES AND TRANSUDATES

**Principles.**—1. Pleural and pericardial transudates are usually sterile when collected aseptically. They are sometimes found contaminated with *Staphylococcus albus* or other organisms from the skin.

2. In pleuritis (*empyema*) the pus collected by aspiration usually shows infection due to pneumococci, hemolytic streptococci, *Staphylococcus aureus* or *B. influenzae*. In chronic pleuritis with drainage organisms of secondary infection or contamination are not unusual as described under the Bacteriological Examination of Fistulae.

3. In pericarditis the exudate or pus usually shows the presence of a streptococcus, pneumococcus or staphylococcus. In pericarditis following trauma additional organisms may be found.

**Method.**—1. Centrifuge if necessary and prepare smears of the sediment. Stain by the method of Gram. If tuberculosis is suspected stain by the method of Ziehl-Neelsen and examine very carefully for acid-fast bacilli.

2. Inoculate plates of blood agar by the surface streak method. Also inoculate tubes of Rosenow's brain broth or glucose hormone broth and incubate at 37° C. for 24 to 48 hours. If tuberculosis is suspected inoculate tubes of special media; inoculate guinea-pigs.

3. Examine colonies. Prepare and stain smears by the method of Gram. Identify organisms present. The following include those usually present:

Gram-positive	{ Staphylococci Streptococci Pneumococci <i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
Gram-negative	{ <i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> ) Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> ) <i>B. influenzae</i> ( <i>Hemophilus influenzae</i> ) <i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> ) <i>B. proteus vulgaris</i>
Acid-fast	<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

## BACTERIOLOGICAL EXAMINATION OF BILE AND GALLSTONES

**Principles.**—1. Bile is best obtained for bacteriological examination by aspiration of the gallbladder at operation or following the surgical removal of the gallbladder.

2. Bile collected by the Lyon method of duodenal drainage is subject to contamination by saliva, by the stomach and duodenum but when collected with rigid attention to technic as described on pages 210 to 215 is usually acceptable for bacteriological examination.

3. At the time of drainage about 20 drops should be added to 150 cc. of hormone broth with a pH of 7.4 to 7.6 suitable for the cultivation of streptococci.

4. Fractions may be collected on sterile vials or test tubes.

**Method.**—1. Centrifuge a portion; prepare smears and stain by method of Gram. The direct examination of wet preparations is also recommended.

2. With a sterile pipet inoculate 0.5 to 1 cc. on blood agar plates by the surface streak method.

3. If a culture in hormone broth has not been made at the time of operation or drainage, inoculate a flask with 0.5 to 1 cc.

4. A duplicate set of cultures for anaerobic cultivation is advisable.

5. Incubate at 37° C. for 24 to 72 hours. Examine colonies. Prepare smears and stain by Gram method. Identify organisms. The following include those generally found in pure or mixed culture:

Gram-positive	{	Staphylococci
	{	Streptococci
	{	<i>M. tetragenus</i> ( <i>Gaffkya tetragena</i> )
	{	<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
	{	<i>B. subtilis</i>
	{	<i>Saccharomyces cerevisiae</i> , etc.
Gram-negative	{	<i>B. coli</i> ( <i>Escherichia coli</i> )
	{	<i>B. typhosus</i> ( <i>Eberthella typhi</i> )
	{	<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
	{	Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> )
	{	<i>B. proteus vulgaris</i>

6. Gallstones may be dipped for a few seconds in boiling water for surface sterilization. Crush in a mortar under rigid aseptic precautions and culture in a flask of plain or glucose hormone broth. Incubate at 37° C. for 24 to 48 hours. If a growth occurs, prepare smears stained by method of Gram. If a mixed growth is present, plate on blood agar by the surface streak method. Incubate at 37° C. for 24 to 48 hours. Examine colonies; prepare smears and stain by Gram method. Identify organisms. Those usually present are included among the organisms listed above.

## BACTERIOLOGICAL EXAMINATION OF THE FECES AND RECTUM

**Principles.**—1. The feces contain so many different varieties of bacteria that a general bacteriological examination is inadvisable.

2. All requests, therefore, should specify the particular organism or organisms to be examined for in order to choose the proper culture media and technic.

3. The method of collection of material for bacteriological examination is important and is described on page 312. In the bacteriological examination of ulcers of the colon and rectum the material should be collected by a proctologist.

4. Mucus and pus are especially desirable for bacteriological examination.

**Method.**—1. Prepare thin smears and stain by the method of Gram. Their only value is an estimated percentage of gram-positive and gram-negative organisms. None of the pathogenic organisms can be identified by smear alone except in the case of tubercle bacilli detected in smears stained by the Ziehl-Neelsen or fluorescent methods.

2. For the detection of bacilli of the typhoid-paratyphoid-dysentery and cholera groups see special methods under the respective organisms.

3. For general bacteriological examination as for streptococci, staphylococci, etc., a loopful of material may be diluted in a tube of broth or sterile saline solution; mix well and transfer 2 or 3 loopsful to a second tube. Prepare blood agar plates from each dilution by the surface streak method. Or a loopful of material may be grown in a tube of Rosenow's brain or hormone broth for 24 hours and used for the preparation of blood agar plates. Incubate at 37° C. for 24 to 48 hours. Study the colonies; prepare smears and stain by the Gram method. Identify the different organisms.

4. For *B. welchii*, *B. tetani* and other pathogenic anaerobic spore forming bacilli use strict anaerobic cultivation for several days.

5. If acid-fast bacilli are found in direct smears or after concentration, inoculate guinea-pigs.



6. The organisms of most interest are as follows:

Gram-positive	{	Staphylococci	
		Streptococci	
		Pneumococci	
		<i>B. welchii</i>	} and other anaerobes
		<i>B. tetani</i>	
	{	<i>B. acuminatus</i> ( <i>Bacterioides acuminatus</i> )	
		<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )	
		<i>B. subtilis</i>	
		<i>B. acidophilus</i> and lactic acid groups	
		<i>B. bifidus</i> ( <i>Bacteroides bifidus</i> )	
	{	<i>Monilia psilosis</i>	
Gram-negative	{	Gonococcus ( <i>Neisseria gonorrhoeae</i> )	
		<i>B. coli</i> ( <i>Escherichia coli</i> )	
		<i>B. cholerae</i> ( <i>Vibrio comma</i> )	
		<i>B. typhosus</i> ( <i>Eberthella typhi</i> )	
		<i>B. paratyphosus A</i> ( <i>Salmonella paratyphi</i> )	
		<i>B. paratyphosus B</i> ( <i>Salmonella schottmuelleri</i> )	
		<i>B. enteritidis</i> ( <i>Salmonella enteritidis</i> )	
		<i>B. dysenteriae</i> ( <i>Shigella dysenteriae</i> )	
		<i>B. faecalis-alcaligenes</i>	
		<i>B. ambiguus</i> ( <i>Shigella ambigua</i> )	
Acid-fast		<i>B. liquefaciens</i> ( <i>Bacteroides liquefaciens</i> )	
		<i>B. proteus vulgaris</i>	
		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )	

## BACTERIOLOGICAL EXAMINATION OF THE URINE

**Principles.**—1. Urine for bacteriological examination should be collected with precautions against contamination. Methods are described on page 316.

2. It is always advisable to state the particular organism or organisms to be examined for in order to properly select special methods for examination.

3. Cultures should be made as soon as possible after collection.

4. Urinary antiseptics should not be administered for 24 to 48 hours previous to the collection of the specimen.

5. A method for examination for tubercle bacilli is given on page 462; for typhoid-paratyphoid bacilli on page 471; for gonococci on page 435. A general examination for other organisms may be conducted as follows:

**Method.**—1. With a sterile pipet transfer 1 cc. of urine to a tube of hormone broth and 0.5 cc. to a plate of blood agar.

2. Centrifuge 5 to 15 cc. under rigid aseptic precautions. Remove the supernatant urine. Prepare smears of the sediment, inoculate blood infusion agar and stain by the method of Gram and for acid-fast bacilli. Inoculate plates of desoxycholate agar with sediment; also a tube of infusion broth. If the patient is receiving sulfonamide therapy, also inoculate broth carrying 5 mg. p-aminobenzoic acid per 100 cc. Incubate at 37° C.

If the plates are sterile, the broth culture may show growth after 24 to 72 hours' incubation, usually due to contaminants. If growths appear prepare smears and stain by the Gram method. Identify organisms if any are present.

3. Normally urine is sterile when aseptically collected, but under pathological conditions any of the following may be present:

Gram-positive	{ Staphylococci Streptococci <i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
Gram-negative	{ Gonococcus ( <i>Neisseria gonorrhoeae</i> ) <i>M. catarrhalis</i> ( <i>Neisseria catarrhalis</i> ) <i>B. proteus vulgaris</i> <i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> ) <i>B. typhosus</i> ( <i>Eberthella typhi</i> ) <i>B. paratyphosus A</i> ( <i>Salmonella paratyphi</i> ) <i>B. paratyphosus B</i> ( <i>Salmonella schottmuelleri</i> ) <i>B. coli</i> ( <i>Escherichia coli</i> ) <i>B. dysenteriae</i> ( <i>Shigella dysenteriae</i> ) <i>B. abortus</i> ( <i>Brucella abortus</i> )
Acid-fast	{ <i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> ) <i>B. smegmatis</i> ( <i>Mycobacterium smegmatis</i> )

### BACTERIOLOGICAL EXAMINATION OF THE UROGENITAL ORGANS AND PROSTATE GLAND

**Principles.**—1. *Spirochaeta pallida* in genital sores is best detected by darkfield examination. Methods for the collection of material are given on page 318.

2. Examinations for *gonococci* are best conducted with smears of pus stained by the Gram method supplemented by cultures. Smears and cultures of urine sediment are sometimes employed but are not usually satisfactory. Vaginal washings are useful and a method for collection is given on page 317.

3. A method for the collection of material for examination for *B. ducrey* (chan-croid) is given on page 320.

4. Material from the prostate gland for bacteriological examination may be obtained by methods given on page 317.

5. Urine collected by catheterization from the bladder or kidneys may be examined for tubercle bacilli as described on page 426; for general examination the method previously given may be employed.

6. The organisms most commonly found in infections of the urogenital organs may be listed as follows:

Gram-positive	{ Staphylococci Streptococci Pneumococci <i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> ) <i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
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Gram-negative	{	Gonococcus ( <i>Neisseria gonorrhoeae</i> )
		<i>B. proteus vulgaris</i>
		Bacillus of Ducrey ( <i>Hemophilus ducreii</i> )
		<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>B. acidophilus</i> ( <i>Lactobacillus acidophilus</i> )
Acid-fast	{	<i>Spirochaeta refringens</i> ( <i>Borrelia refringens</i> )
		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )
		<i>B. smegmatis</i> ( <i>Mycobacterium smegmatis</i> )

### BACTERIOLOGICAL EXAMINATION OF THE BLOOD

**Principles.**—1. Bacteria are but rarely found in direct smears of the blood. Cultures are required and the technic is very important.

2. Anaerobic cultures are frequently advisable and especially in suspected streptococcus and *Cl. welchii* infections.

3. Bacteremia may be present without demonstrable clinical manifestations, but the number of organisms present is apt to be small, requiring the cultivation of relatively large amounts of blood.

4. The complement and natural antibodies of the blood may inhibit the growth of small numbers of organisms. For this reason the blood should be sufficiently diluted with culture medium. No culture should be finally reported as sterile unless incubation has been conducted for at least 10 days and in some instances for 14 to 21 days.

5. Postmortem blood cultures of the heart should be made within an hour after death, before general bacterial invasion of the tissues has occurred.

6. Organisms producing large amounts of exogenous toxins like the diphtheria and tetanus bacilli rarely produce septicemia. *B. welchii* is sometimes found, but usually just before death or postmortem.

7. *Spirochaeta pallida* may be found during the acute early stages of syphilis, but is only detected by the intratesticular inoculation of rabbits. *Spirochaeta recurrentis* may be detected by darkfield examination of the blood or by the examination of stained smears.

8. Staphylococci, diphtheroid bacilli, *B. subtilis*, *B. coli* and other organisms from the skin may contaminate blood cultures. When any of these are found, the culture should be repeated.

9. For routine purposes, when infections with aerobic streptococci, staphylococci, pneumococci, meningococci, gonococci, *B. coli*, *B. typhosus* or *B. influenzae* are suspected, 15 to 20 cc. of blood may be collected with scrupulous aseptic precautions and placed in a small flask carrying 3 cc. of a sterile 2 per cent solution of sodium citrate. Mix immediately to prevent coagulation. Melt 2 tubes of infusion agar and cool to 45° C. With a sterile pipet add 1 cc. of the citrated blood to one tube and 2 cc. to the second. Mix and pour into sterile Petri dishes. Transfer the remainder of the blood to 100 cc. of infusion broth. If the patient has received sulfonamide therapy both the agar and broth should carry 5 mg. of p-aminobenzoic acid per 100 cc. Incubate at 37° C. and examine the cultures daily. Often growth will appear in the broth flask while the plates remain sterile. In such instances streak a blood agar plate with a small loopful of the material. After 6 days' incubation all blood cultures which have shown

no growth should be subcultured routinely by streaking a loopful of the well shaken broth culture on a blood agar plate. This procedure may result in the detection of organisms present in such small numbers that they might otherwise be overlooked.

10. If anaerobic streptococci or *Cl. welchii* are suspected inoculate 2 tubes of Brewer's thioglycollate broth with 1 to 2 cc. of citrated blood. Incubate aerobically at 37° C. for 8 to 10 days examining daily for growth. Otherwise, routine cultures may be incubated in an anaerobic jar.

11. If Brucella infection is suspected, collect 10 cc. of blood in 10 cc. of sterile 4 per cent sodium citrate in saline solution and incubate at 37° C. for at least 20 days in an atmosphere of a 10 per cent carbon dioxide, as recommended by Gould and Huddleson (*J.A.M.A.*, 109: 1971, 1937).

12. *B. tularensis* is difficult to cultivate. Ransmeier and Schaub (*Arch. Int. Med.*, 68: 747, 1941) advise inoculating 6 or 7 slants of glucose-cystine blood agar with 0.5 to 1 cc. of blood (preferably not citrated). Incubate aerobically at 37° C. for 4 to 10 days, or until growth is observed (do not report negative before 3 weeks' incubation).

13. In all blood cultures prepare smears if and when growths appear and stain by the Gram method. Identify the organisms. It is a good practice to submit reports at the end of 24 hours' incubation and at frequent intervals thereafter until conclusively negative results are observed. The number of colonies on plates may be reported in terms of the number per cc. of blood plated.

14. The bacteria most commonly encountered in blood cultures are as follows:

Gram-positive	{	Staphylococci
		Streptococci
		Pneumococci
		<i>B. anthracis</i>
		<i>Cl. welchii</i> , etc.
Gram-negative	{	Meningococcus ( <i>Neisseria intracellularis</i> )
		Gonococcus ( <i>Neisseria gonorrhoeae</i> )
		<i>B. abortus</i> ( <i>Brucella abortus</i> )
		<i>B. typhosus</i> ( <i>Eberthella typhi</i> )
		<i>B. paratyphosus A</i> ( <i>Salmonella paratyphi</i> )
		<i>B. paratyphosus B</i> ( <i>Salmonella schottmuelleri</i> )
		<i>B. dysenteriae</i> ( <i>Shigella dysenteriae</i> )
		<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		Friedländer's bacillus ( <i>Klebsiella pneumoniae</i> )
		<i>B. influenzae</i> ( <i>Hemophilus influenzae</i> )
		<i>B. proteus vulgaris</i>
		<i>B. pestis</i> ( <i>Pasteurella pestis</i> )
Acid-fast		<i>Spirochaeta obermeirei</i> ( <i>Borrelia recurrentis</i> )
		<i>Spirochaeta novyi</i> ( <i>Borrelia novyi</i> )
		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )

**BACTERIOLOGICAL EXAMINATION OF THE SKIN**

**Principles.**—1. Many different kinds of organisms may be found on the skin in health and disease.

2. Requests should specify the kind of examination desired to determine the proper selection of methods, culture media, etc.

3. The bacteria most commonly encountered are as follows:

Gram-positive	{	Staphylococci
		Streptococci including <i>Streptococcus scarlatinae</i> and <i>Streptococcus erysipelatis</i>
		Pneumococci
		<i>B. diphtheriae</i> ( <i>Corynebacterium diphtheriae</i> )
		<i>B. pseudodiphtheriae</i> ( <i>Corynebacterium pseudodiphtheriae</i> )
		<i>B. acnes</i> ( <i>Corynebacterium acnes</i> )
		<i>B. anthracis</i>
		<i>Erysipelothrix</i> ( <i>Erysipelothrix rhusiopathiae</i> )
		Actinomyces
		<i>Actinomyces hominis</i>
		<i>Actinomyces madurae</i>
		Sporotrichia ( <i>Sporotrichum beurmanni</i> )
		Blastomycetes
Gram-negative	{	<i>B. pyocyaneus</i> ( <i>Pseudomonas aeruginosa</i> )
		<i>B. mallei</i> ( <i>Pfeifferella mallei</i> )
		<i>B. coli</i> ( <i>Escherichia coli</i> )
		<i>B. catarrhalis</i> ( <i>Neisseria catarrhalis</i> )
		<i>B. proteus vulgaris</i>
Acid-fast	{	<i>Spirochaeta vincentii</i>
		<i>B. tuberculosis</i> ( <i>Mycobacterium tuberculosis</i> )
		<i>B. leprae</i> ( <i>Mycobacterium leprae</i> )
		<i>B. smegmatis</i> ( <i>Mycobacterium smegmatis</i> )

4. All local treatment and especially with germicidal agents should be omitted for at least 24 hours before bacteriological examinations are made.

5. A method for the collection of material for examination for *B. leprae* is given on page 320.

6. For other organisms including the yeasts, molds and fungi, scrapings with a sterile scalpel are desirable. Mere surface swabbings are of little or no value. Pieces of skin aseptically removed by biopsy are sometimes required.

**POSTMORTEM BACTERIOLOGICAL EXAMINATIONS**

1. Terminal and postmortem invasion of the blood stream and organs greatly increases the number and kinds of organisms which may be found. Consequently, bacteriological examinations must be made as soon as possible after death and before embalming.

2. The primary infecting organism does not usually occur in pure culture, numer-

ous secondary invaders often accompanying it, especially *B. coli*, *B. aerogenes* and *B. proteus vulgaris*.

3. Smears should be made and examined routinely to determine the approximate number of organisms present.

4. Cultures of organs are best taken with sterile capillary pipets and rubber bulbs. The surface of the organ should be seared with a small, hot soldering iron or knife in an area about 1 inch square. A puncture is then made through the sterile area with a fairly wide-mouthed pipet, and as much material as possible obtained and discharged immediately into infusion broth. A drop of the material should be used for preparing smears.

5. For cultures from wounds, abscesses, ears, meninges, etc., use sterile swabs.

6. In bacterial endocarditis, obtain a small piece of a friable vegetation with sterile scissors and forceps. Wash the tissue by passing it through 5 or 6 tubes of warm sterile saline solution. Transfer to a tube of infusion broth and crush against the sides of the tube by means of a sterile glass rod with a sharp, jagged end.

7. Infusion broth cultures should be plated out immediately on blood infusion agar pour plates. It is also advisable to add sterile blood to the infusion broth cultures and incubate at 37° C.

8. If enteric organisms are suspected, prepare plates of desoxycholate agar and desoxycholate citrate agar.

9. For *H. pertussis* prepare plates of the Bordet-Gengou potato blood medium.

10. In lung and liver abscesses, uterine cultures, and others where anaerobic streptococci, *Cl. welchii* or other anaerobic infections are suspected, inoculate plates of blood agar and potassium tellurite agar and incubate in an anaerobic jar. Also inoculate Brewer's thioglycollate medium and incubate aerobically.

11. If blastomycosis, actinomycosis or sporotrichosis is suspected, inoculate 4 plates of Sabouraud's agar. Incubate 2 at room temperature and 2 at 37° C. Observe for 10 days to 2 weeks before discarding the plates as negative.

## BAACTERIOLOGICAL EXAMINATION OF SULFONAMIDE POWDERS

A method recommended by Landy and Oswald (*Jour. Lab. and Clin. Med.*, 28: 743, 1943) is as follows:

1. Aseptically transfer 5 grams of the powder to a flask containing 500 cc. of sterile water.

2. Shake vigorously for 5 minutes, allow to stand for 30 minutes at room temperature, and shake again for 5 minutes.

3. Allow the undissolved powder to settle.

4. Transfer 5 cc. of the supernatant fluid to each of 2 tubes containing 20 cc. of Brewer's sodium thioglycollate broth. This medium suffices for the cultivation of *Cl. tetani* and other anaerobic organisms as well as for staphylococci and *B. coli*.

5. Incubate the tubes aerobically at 37° C. for a period of 7 days and examine at intervals.

**METHODS FOR THE IDENTIFICATION OF STAPHYLOCOCCI**

1. Staphylococci may occur singly, in pairs or in very short irregular chains but usually in grape-like clusters (Fig. 167); rarely in packets.

2. They stain readily and are gram-positive, but in both smears of pus and cultures gram-negative cocci may occur. They are not encapsulated.

3. They grow readily in all ordinary culture media. On solid media the colonies are circular and opaque with smooth glistening surfaces and even edges. On blood agar many strains and especially *Staphylococcus aureus* produce zones of hemolysis. In broth they produce uniform turbidity with moderate deposits readily disintegrating on shaking. Gelatin is usually liquefied in 5 days at 22° C. and milk coagulated.

4. Some staphylococci are nonhemolytic in both deep and surface colonies; this is characteristic of most strains of non-pathogenic *Staphylococcus albus*; others are hemolytic both surface and deep which is characteristic of virulent *Staphylococcus aureus*. The majority of strains, however, produce no hemolysis around deep colonies but are hemolytic on the surface of blood agar plates. This is a characteristic of staphylococci which is not shown by other bacteria.

5. Differentiation between hemolytic staphylococci and beta hemolytic streptococci on the surface of blood agar plates is sometimes difficult. The colonies of staphylococci are large, soft and opaque with a narrow hemolytic zone. The colonies of streptococci are smaller, translucent and hard, with zones of hemolysis extending well beyond the edges.

6. After 2 or 3 days of aerobic cultivation on solid media, pigments may be produced in the colonies which are of great value in identifying the type:

- (a) Golden-orange: *Staphylococcus aureus*
- (b) Lemon-yellow: *Staphylococcus citreus*
- (c) No pigment (white): *Staphylococcus albus*

7. Identification and differentiation can usually be made by these means. Always report the type present and whether hemolytic or nonhemolytic.

8. *Staphylococcus epidermidis albus* is merely a special type found on the skin and in "stitch abscesses"; it ferments sucrose, but not mannitol and raffinose, whereas *Staphylococcus albus* ferments sucrose and mannitol but not raffinose.

9. *Staphylococcus aureus* usually produces several filterable toxins now commonly converted into toxoids for purposes of active immunization. These are secured by cultivation in hormone broth (pH 7.4 to 7.6) for 5 days, followed by Berkefeld filtration. The filtrate may contain the following: (1) hemolysin especially active for rabbit erythrocytes; (2) leukocidin; (3) a necrotizing toxin best detected by intra-

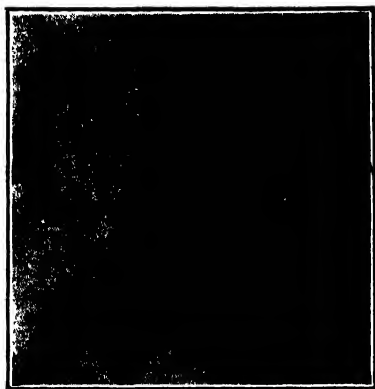


FIG. 167.—*STAPHYLOCOCCUS AUREUS*  
Gram stain.  $\times 1200$ .

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

cutaneous injection of rabbits; (4) a lethal toxin, and (5) an enterotoxin concerned in food poisoning.

**Virulence of Staphylococci; Coagulase Test.**—It is frequently necessary to determine whether or not a staphylococcus is virulent, especially in the case of strains recovered from cultures of the blood or urine. The test of choice is inoculation of mice or rabbits, but is apt to be too expensive and time-consuming.

Practically all virulent staphylococci are hemolytic, produce pigment, and give a positive coagulase reaction. The latter is particularly important and is conducted as follows: (a) Place 0.5 cc. of citrated or oxalated blood plasma (usually rabbit) in a small test tube; (b) inoculate with 1 large loopful of culture from a plain or blood agar slant or plate; (c) place in a water bath at 37° C. for 1 to 3 hours; (d) the production of a small kernel of coagulum is a weakly positive reaction; complete coagulation is strongly positive.

**Cat Test for Staphylococcus Enterotoxin.**—This test is of value in determining the production of enterotoxin produced by staphylococci recovered in cultures of foods in suspected staphylococcus food poisoning. The technic after that of Hammon (*Am. Jour. Pub. Health*, 31: 1191, 1941) may be as follows:

1. Cultivate the staphylococcus in a tube of hormone broth for at least 5 days.
2. Place in a boiling water bath for 30 minutes (enterotoxin is thermostable).
3. Centrifuge thoroughly and use the supernatant fluid.
4. Inject a *healthy* cat intravenously (saphenous vein) with 0.5 to 5.0 cc. (ordinarily 2.0 cc.); anesthesia is not required. Give the animal a moderately sized meal shortly before the injection. Take rectal temperature before injection.
5. The presence of enterotoxin is indicated by vomiting in 15 minutes to 2 hours (usually about 30 minutes) with coarse tremors (chills) followed by fever in 2 to 4 hours. Mild diarrhea usually occurs and persists for several hours. After 3 to 4 hours, the cat begins to recover and in 24 to 48 hours appears to be normal.

## METHODS FOR THE IDENTIFICATION OF STREPTOCOCCI

1. Streptococci occur in chains of variable length (Fig. 168); the longest chains are always observed in broth cultures. On solid media the chains are usually short and sometimes in diplococcus formation, requiring differentiation from pneumococci. They are sometimes encapsulated.

2. They stain readily and are usually gram-positive. However, gram-negative elements may occur and some saprophytic streptococci are entirely gram-negative.

3. On blood agar the streptococci are divisible into (a) beta or hemolytic streptococci (Fig. 169); (b) gamma or nonhemolytic streptococci and (c) alpha or viridans streptococci.

4. The surface colonies of *beta streptococci* are usually small, white, hard, rather opaque and surrounded by a well-defined clear zone of hemolysis (Plate IX) due to the production of erythrogenic toxin. However, if the organisms have retained their capsules, the colonies will appear larger, more translucent, mucoid, with a tendency to become confluent. Occasionally the colonies are rough and very hard with definite greenish discoloration.

These streptococci have been divided into groups A, B, C, D, E, F, G, H and K by Lancefield (*Jour. Exper. Med.*, 47: 91, 469 and 857, 1928; *ibid.*, 57: 571, 1933) by



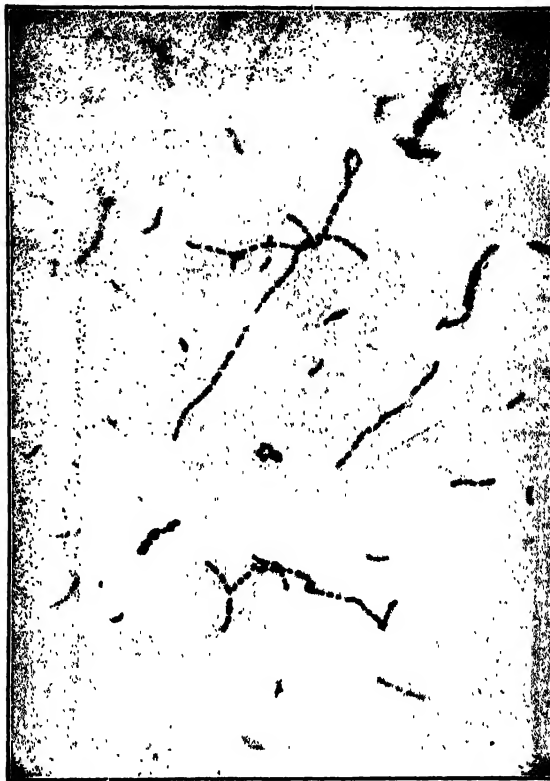


FIG. 168.—STREPTOCOCCUS PYOGENES

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

means of precipitin reactions employing "C" (carbohydrate) antigens. Only streptococci of groups A, B, C and D, however, are pathogenic or potentially pathogenic for man. Of these the streptococci of group A are by all odds the most important in the production of disease (scarlet fever, erysipelas, pneumonia, meningitis, puerperal sepsis, cellulitis and lymphadenitis, etc.).

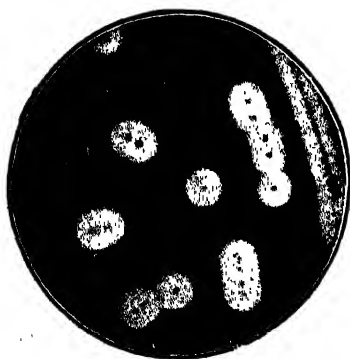


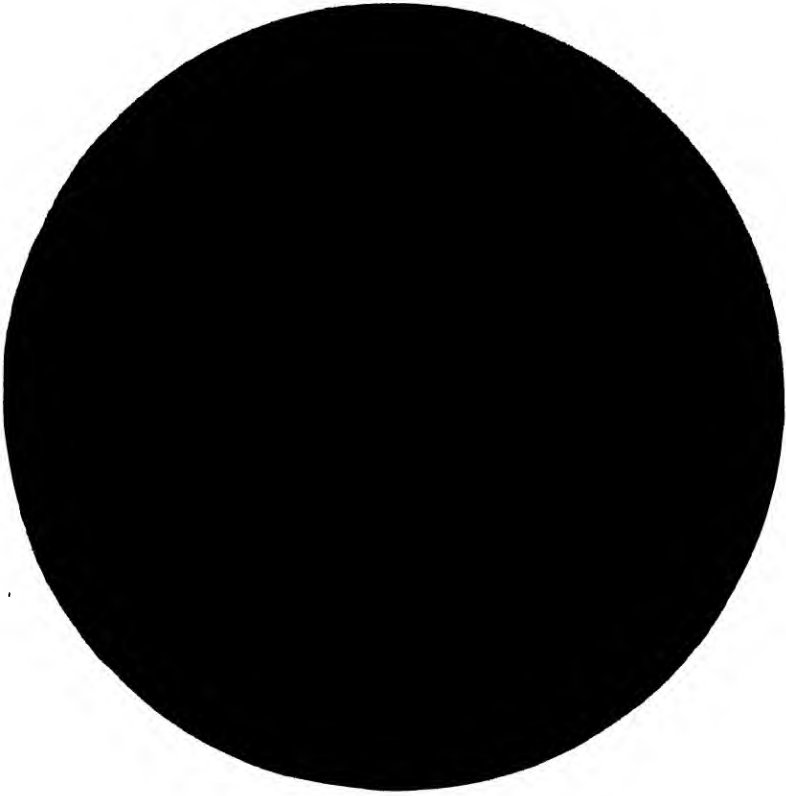
FIG. 169.—ZONES OF HEMOLYSIS AROUND COLONIES OF HEMOLYTIC STREPTOCOCCI

(From Park, Williams and Krumwiede, *Pathogenic Microorganisms*, Lea and Febiger, Philadelphia.)

The deep colonies differ considerably according to their serological group. Colonies of groups A, C and G are very opaque and always in the center of hemolytic zones. Group B colonies are smaller with less clearly defined hemolysis. Group D colonies are characterized by very large zones of hemolysis. Group F colonies are exceptionally small with tiny areas of complete hemolysis.

If an additional test for hemolysis is required, add 0.5 cc. of a broth culture to 0.5 cc. of a 5 per cent suspension of washed erythrocytes (rabbit preferred) in a small test tube and place in a water

PLATE IX



COLONIES OF STREPTOCOCCI ON BLOOD AGAR

*A*, colony of hemolytic streptococcus; *B*, colony of nonhemolytic streptococcus;  
*C*, colony of streptococcus of the viridans type.



bath at 37° C. for 2 hours. Examine at intervals for hemolysis. Centrifuging the tube is helpful for detecting slight hemolysis.

In the older literature hemolytic streptococci were subdivided on the basis of their clinical sources and their fermentation of various carbohydrates into supposed species under the names *Str. scarlatinae*, *Str. erysipelatis*, *Str. anginosus*, etc. Recent work has shown, however, that these strains are not specific for the disease conditions from which they have been isolated; on the contrary, one and the same strain of streptococcus can cause sore throat, scarlet fever, and puerperal fever in different subjects, depending upon individual susceptibility and the circumstances of infection. It is advisable, therefore, not to perpetuate the errors of the past by reproducing the older classification in these terms.

5. The surface colonies of *gamma streptococci* are usually small, gray and translucent with no zones of hemolysis or greenish discoloration (Plate IX). A streptococcus should not be regarded as nonhemolytic unless it has been subcultured at least twice or grown anaerobically. Aerobic gamma streptococci are rarely encountered in routine diagnostic bacteriology. Most strains of streptococci which appear gamma in 24 hours will produce alpha hemolysis if the plates are reincubated for 24 hours and then refrigerated overnight. Anaerobic gamma streptococci, however, occur very frequently.

6. Both surface and deep colonies of *alpha streptococci* vary considerably in their appearance. The greatest differences are observed between strains of respiratory tract and of intestinal origin.

Surface colonies of respiratory strains are small, raised, convex and opaque. They are surrounded by very narrow zones of hemolysis which may or may not show green discolorization due to oxidation. Deep colonies vary tremendously. Some show narrow zones of hemolysis surrounded by an area of greening. Others show little or no discernible hemolysis. Colonies of B to G beta streptococci may resemble those of alpha streptococci but if hemolysis is more pronounced than greening, they may be designated as beta; if greening is more pronounced, they may be designated as alpha. The viridans and nonhemolytic types each contain many different species. For practical purposes, however, it is sufficient to designate them as viridans and nonhemolytic streptococci. The designation of "*Streptococcus viridans*" is incorrect as there is no such species.

Alpha streptococci of enteric origin belong to the *enterococcus group* and, according to their sugar fermentations, are usually alpha *Streptococcus fecalis*. The surface colonies, after 24 hours' incubation, are usually much larger than those of other streptococci and are gray, shiny, more or less translucent, dew-drop colonies, producing no hemolysis or discoloration. Consequently, they may be mistaken for *Staphylococcus albus* or *B. coli*. After 48 hours' incubation they usually show slight green discoloration. Deep colonies are quite large and gamma in appearance, with no hemolysis or greening in 24 hours. After incubation for 48 hours, definite greening is usually observed. Generally no hemolysis is produced until the plates have been refrigerated for another 24 hours. These streptococci may be found in blood cultures in bacterial endocarditis. They are characterized by being able to grow in salt infusion broth (ordinary meat infusion broth with a total of 6.5 per cent sodium chloride). All other streptococci fail to grow in this broth medium.

7. Anaerobic cultures should be made in cases of puerperal sepsis, gangrene of the skin, wound infections following operations upon the upper gastro-intestinal tract

and appendix, suppurative pneumonitis and empyema. There are many micro-aerophile types between aerobic and anaerobic types of streptococci.

8. In broth the growth is rarely diffuse; turbidity is usually absent or slight. A fine granular growth occurs with sediment on the bottom and sides sometimes difficult to break up.

9. The beta-hemolytic streptococci of group A pathogenic for man produce a final pH of 4.6 or higher in dextrose broth, ferment lactose and trehalose but do not ferment sorbitol or hydrolyze sodium hippurate.

10. For differentiating streptococci from pneumococci, add 0.1 cc. of clear ox bile to 0.5 cc. of a pure broth culture and place in a water bath at 37° C. for an hour. Or, add 4 drops of a 10 per cent aqueous solution of sodium desoxycholate to 2 cc. of broth culture and place in a water bath at 37° C. Streptococci are not soluble while most pneumococci go into solution in 5 minutes or less.

11. Also inoculate a tube of Hiss' serum water litmus inulin medium and incubate at 37° C. for 24 to 48 hours. Streptococci do not usually produce acid and thereby change the color of the medium to pink or pinkish-blue, while most pneumococci produce acid with this color change.

12. In case of doubt, conduct typing tests with antipneumococcus sera (see below). This is the quickest method for differentiating streptococci from pneumococci.

**Lancefield's Method for Grouping Hemolytic Streptococci.**—With the exception of group C, only group A beta streptococci regularly produce large zones of clear hemolysis around their colonies. Streptococci of the other groups are characterized by narrow, incomplete or indefinite zones of hemolysis. The outstanding need in clinical bacteriology is a simple and rapid method for grouping these streptococci, permitting at least the differentiation of group A colonies from those of the other groups. The method of Lancefield may be described as follows although it is not a practical routine procedure.

1. Add 50 cc. of N/10 hydrochloric acid to the organisms obtained by centrifuging 50 cc. of an 18-hour neopeptone broth culture. Boil the suspension for 10 minutes, cool rapidly, and centrifuge. Remove and neutralize the supernatant fluid, centrifuge, and then pour the supernatant fluid into 4 volumes of 95 per cent alcohol. The resulting precipitate is separated by centrifugation, and heated in a boiling water bath until all traces of alcohol disappear. This material is the "C" antigen.

2. Dissolve the antigen in 50 cc. of saline solution, and then make 3 serial dilutions, decreasing the concentration of the antigen 10 times for each successive dilution. Add 0.2 cc. of each antigen dilution to successive 0.2 cc. portions of each unadsorbed group A rabbit antiserum. Place in the water bath at 37° C. for 30 minutes, and then in the ice-chest overnight. One or more of the tubes containing the hemologous antiserum and the antigen will show a hard white precipitate.

**Tillet and Garner's Test for Fibrinolysis by Hemolytic Streptococci.**—This test (*Jour. Exper. Med.*, 58: 485, 1933) may be conducted as follows:

1. To 0.2 cc. of oxalated plasma of the patient add 0.8 cc. of sterile saline solution and 0.5 cc. of broth culture or sterile filtrate; mix.

2. Add 0.25 cc. of a 0.25 per cent solution of calcium chloride.

3. Mix and place in a water bath at 37° C.

4. The time at which solid coagulation occurs is noted.

5. Continue to observe at intervals and note time at which complete dissolution

of the clot (fibrin) occurs. All tests in which the plasma clot is resistant to dissolution after 24 hours' incubation are arbitrarily terminated. The plasma of 2 or 3 normal individuals should be tested in the same manner as controls.

6. The plasma clots of patients recovered from hemolytic streptococcus infections are usually highly resistant to dissolution (fibrinolysis).

**Mouse Inoculation Test.**—1. Inject a mouse intraperitoneally with 1 to 0.5 cc. of 24-hour broth culture. Beta-hemolytic streptococci of group A usually produce a fatal septicemia.

2. Inject a mouse intraperitoneally with 1 or 2 cc. of blood immediately after aspiration from a vein of the patient. Virulent streptococci, if present, usually produces a fatal peritonitis and septicemia. Stain smears of heart blood by method of Gram. The presence or absence of capsules aids in differentiation from pneumococci.

### METHODS FOR THE IDENTIFICATION OF *M. TETRAGENUS*

1. This organism is commonly found in the sputum and especially in tuberculosis and other chronic infections.

2. In smears of sputum and secretions it occurs as 4 cocci surrounded by a pseudo-capsule (tetrads) while in cultures on ordinary media it usually occurs in pairs and irregular masses with no capsules (Fig. 170).

3. Gram-positive.

4. On agar plates the colonies are circular, white or grayish-white, smooth, glistening and glutinous, often adherent to the medium and difficult to emulsify. On blood agar the colonies are surrounded by a narrow zone of greenish discoloration (alpha type of hemolysis). In broth there is even turbidity with a later thick, glutinous deposit and comparatively clear supernatant medium.

5. These characteristics are usually sufficient for identification. It commonly produces acid in glucose, maltose, lactose and sucrose. No indol; nitrates are reduced.

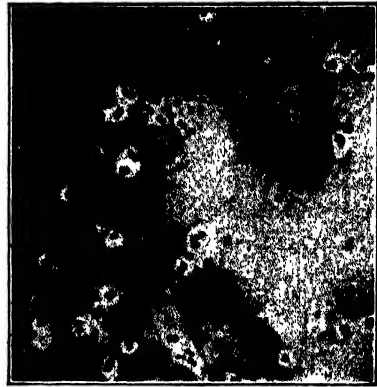


FIG. 170.—*MICROCoccus TETRAGENUS*

(From Zinsser and Bayne-Jones, *Text-book of Bacteriology*, D. Appleton-Century Co., New York.)

### METHODS FOR THE IDENTIFICATION OF PNEUMOCOCCI

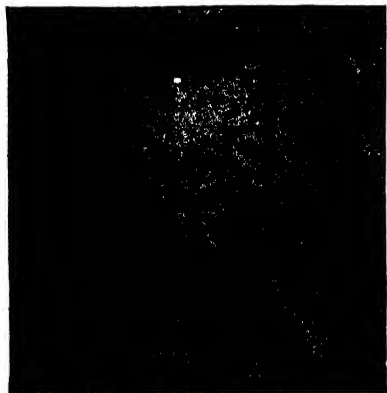
1. If sputum from pneumonia is being examined, smears should be prepared of blood-tinged ("rusty") portions on slides. If cerebrospinal fluid is being examined, centrifuge and prepare smears of the sediment. The pus of empyema or other exudates (nose, ears, etc.) may be prepared in smears.

2. Dry in air; fix by gentle heating and stain by the method of Gram.

3. The pneumococcus is gram-positive and occurs in pairs or, less frequently, in short chains (must be differentiated from streptococcus). The adjacent ends of the cocci are usually rounded while the opposite ends are more pointed or lancet-shaped. A well-marked capsule is present (Fig. 171) which may be retained in cultures on

suitable media. Type III pneumococcus may often be identified by very large capsules. Special capsule stains may be employed.

4. With a platinum loop streak out 1 or 2 blood agar plates with material to be examined. Incubate 24 to 48 hours. Examine colonies; prepare smears and stain by method of Gram. Or culture by the following method of Avery: Select a tenacious portion of the sputum about the size of a bean, and wash 3 or more times in sterile saline solution. Grind the washed sputum in a sterile mortar with about 1 cc. of sterile broth to secure a homogeneous suspension. Inoculate a tube of Avery broth with about



**FIG. 171.—PNEUMOCOCCI WITH STAINED CAPSULES**

(From Zinsser and Bayne-Jones, *Text-book of Bacteriology*, D. Appleton-Century Co., New York.)

0.2 cc. In the case of spinal, pleural or other fluids inoculate with 1 cc. Incubate at 37° C. for 5 to 8 hours. If a good growth has occurred, centrifuge at low speed for 2 minutes to throw down the erythrocytes. Inoculate blood agar plates with the supernatant bacterial suspension and incubate for 24 to 48 hours. With the balance prepare smears and stain by the method of Gram. If pneumococci are present in practically pure culture the suspension may be preserved and used, when occasion arises, for bile solubility, agglutination and precipitation tests.

5. Another method for the rapid isolation of pneumococcus from sputum is as follows: (a) Inoculate a mouse intraperitoneally with 1 cc. of the emulsion of washed sputum prepared as described above. (b) Usually in from 4 to 48 hours, the animal becomes ill and succumbs (the time varies according to numbers and virulence of pneumococci present). (c) When ill kill the mouse or immediately after death, remove the peritoneal exudate aseptically with a capillary pipet. Prepare smears and stain by Gram method. Inoculate glucose hormone broth. Also prepare smears and culture of heart blood. The balance of the peritoneal exudate may be used for agglutination tests as described on page 431 for determination of type of pneumococcus.

6. Surface colonies of pneumococci on blood agar plates are quite characteristic and easily recognized. They are small, flat, shiny, usually green and transparent. Due to a raised edge and heaped up center, they give the appearance of growing in 2 concentric rings. The colonies may be surrounded by an area of green discoloration or a small zone of apparently clear hemolysis. They are readily differentiated from colonies of alpha streptococci which are small, raised, convex and opaque. Colonies of type III pneumococcus are very large, green, raised, mucoid, confluent and have the appearance of drops of oil. After 24 hours' incubation, due to the loss of capsules, they dry down and become quite flat and, except for being much larger, give the characteristic appearance of the usual pneumococcus colony.

The deep colonies of pneumococci are identical with those of alpha streptococci and it is impossible to differentiate between them.

Therefore, since pneumococci produce both hemolysis and "greening" of blood agar, they may be confused either with hemolytic or viridans streptococci. Early in its

growth on blood agar the pneumococcus resembles the hemolytic streptococcus. An older culture shows definite "greening" which may entirely mask the earlier hemolysis and, therefore, be mistaken for a viridans streptococcus.

7. Inoculate a tube of glucose hormone broth and a tube of Hiss' serum water litmus inulin medium with pure colonies.

8. Incubate 24 hours. Examine smears stained by Gram method. Pneumococci usually produce acid and coagulation of the inulin medium with a change of color from blue to pink; streptococci do not.

9. If the broth culture shows a pure growth, conduct a bile solubility test by adding 4 drops of a 10 per cent aqueous solution of sodium desoxycholate to 2 cc. of culture in a small test tube. Place in a water bath at 37° C. Typical pneumococci go into solution in 2 to 5 minutes; typical streptococci do not undergo dissolution. Bayliss (*Jour. Lab. and Clin. Med.*, 28: 748, 1943) recommends adding 0.1 cc. of a 2 per cent solution of crude sodium lauryl sulfate ("Dreft") to 0.9 cc. of a 24-hour culture. Usually the results may be determined in a few minutes but if clearing does not occur, it is necessary to incubate the mixture at 37° C. for ½ hour before the final reading.

**Methods for the Typing of Pneumococci.**—Pneumococci are divisible into 33 or more serologic types. The most virulent are types II and III. The latter has a tendency to grow in chains and develop very large capsules; at one time it was known as *Streptococcus mucosus*. Without doubt, typing is the quickest method of differentiating pneumococci from streptococci.

1. Typing may be conducted with sputum, pleural and peritoneal exudates, cerebrospinal fluid and cultures of these as well as of the blood and fauces, if sufficient numbers of pneumococci are present.

2. Fresh sputum is preferred but, if kept at a low temperature, is satisfactory even when several days old.

3. Blood cultures and spinal fluids showing sufficient numbers of pneumococci should be briefly centrifuged to throw down cells and the supernatant fluid employed.

4. If sputum, spinal fluid and exudates contain too few pneumococci, inoculate tubes of a suitable broth medium and incubate at 37° C. for 8 to 24 hours when sufficient numbers of the organism are usually present. Otherwise, inoculate mice intraperitoneally; in 4 to 12 hours the peritoneal exudates usually contain sufficient pneumococci.

5. Biological laboratories supply 6 pooled sera each containing several type sera. Conduct tests by the Neufeld "quellung" method with these 6 pooled sera. If negative reactions are observed with all, the organism is not at all likely to be a pneumococcus. If any pooled serum gives a positive reaction, it is then necessary to repeat the tests with each separate type specific serum contained in the pool to determine the type of pneumococcus. Monovalent or type specific sera are obtainable for the 32 or 33 specific types of pneumococci for this purpose.

**Neufeld "Quellung" Method.**—1. For each serum, place a small fleck of sputum ("rusty" portions preferred) or a loopful of other material on a slide. When the sputum is very tenacious it may be dislodged with a wire loop or small portions cut off with a flamed scissors. The number of pneumococci per microscopic field should not be excessive as otherwise a typical reaction may not occur.

2. Add a small drop of serum and mix thoroughly. If the serum is not colored



with methylene blue, add a loopful of a 1:5 aqueous dilution of Löffler's methylene blue and mix thoroughly.

3. Put a coverglass on each mixture and press firmly with a blunt object to produce thin preparations.

4. Keep at room temperature and after a few minutes, examine each preparation with an oil immersion objection with the diaphragm partly closed. An artificial blue light is advantageous. The light should not be too subdued, but of sufficient intensity to prevent shadows and the appearance of artefact halos around the organisms.

5. A positive reaction is indicated by a swelling of the capsules which take on a ground-glass appearance with a sharp outline (Fig. 172). The sharpness of the outline is perhaps of more diagnostic value than the degree of swelling. If no reaction occurs

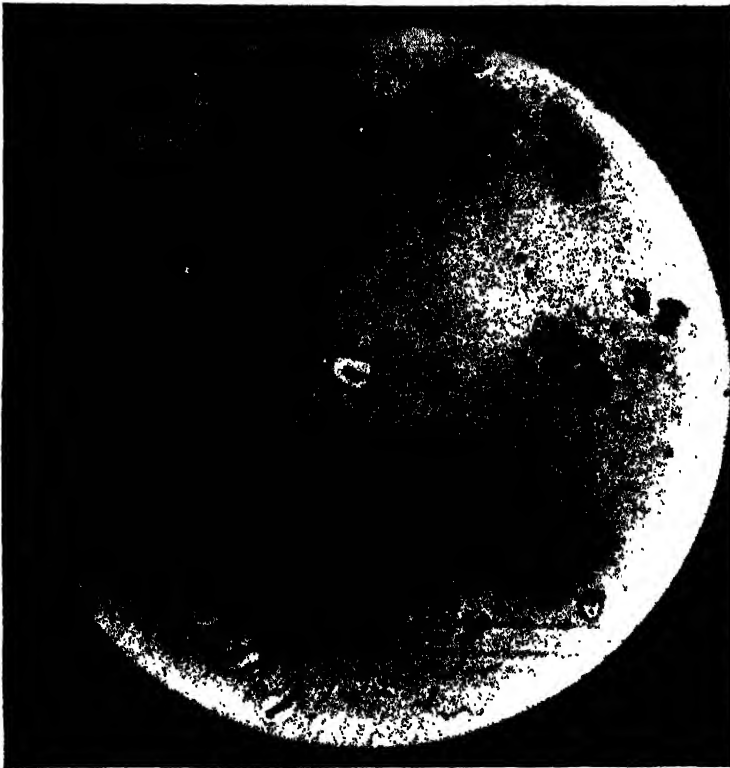


FIG. 172.—THE NEUFELD "QUELLUNG" REACTION FOR THE TYPING OF PNEUMOCOCCI  
( $\times 1500$ ) BULLOWA AND WILCOX

(Courtesy of Lederle Co.)

within a few minutes, the preparations should be examined again at the end of 10 minutes. If type III pneumococci are present, the organisms may appear in large masses without definite capsular swelling. In such cases it may be necessary to repeat the tests with type III serum diluted 1:4 with saline solution.

**Avery, Chickering, Cole and Dochez Agglutination Method.**—1. If the sputum is very fluid and has no firm portions, inoculate a mouse directly. Otherwise, take up a

firm portion consisting of not more than 0.5 cc., in a sterile glass syringe without a needle and wash it 3 times in sterile salt solution. Grind the washed sputum in a sterile mortar, adding from 1.5 to 2.5 cc. of broth during the process (Figs. 173 and 174).

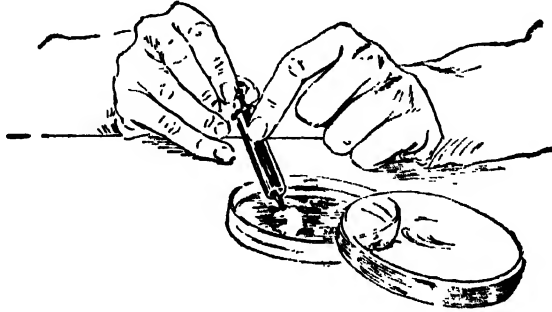


FIG. 173.—WASHING SPUTUM IN SALT SOLUTION

(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

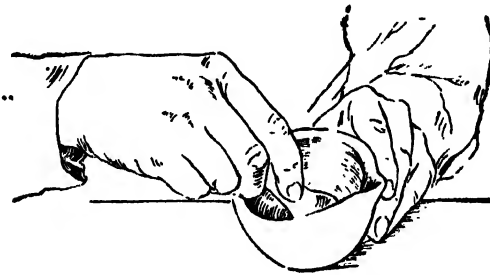


FIG. 174.—EMULSIFYING SPUTUM IN BROTH

(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

2. Inoculate a mouse intraperitoneally with from 0.5 cc. to 1 cc. of the diluted, washed sputum.

3. Within from 4 to 8 hours, puncture the peritoneum of the mouse with a sterile needle attached to a syringe and withdraw 1 to 2 drops of exudate. Spread this on a slide and stain by Gram's method. If microscopic examination shows as many gram-positive cocci as may be found in an 18-hour broth culture, chloroform the mouse and proceed with the test.

4. Since it is desired to recover the organisms in pure culture, carefully observe sterile precautions throughout the autopsy.

5. When the skin has been laid back, make a short, longitudinal opening in the abdominal wall (Fig. 175). Take a loopful of the peritoneal exudate and streak half a blood agar plate. Then enlarge the opening in the abdominal wall and note if the exudate is sticky, suggesting the presence of type III pneumococci or *B. mucosus-capsulatus* (Friedländer's bacillus). Using a bulb pipet, wash the peritoneum thoroughly with from 3 to 4 cc. of salt solution, and put the washings in a centrifuge tube. Then, with sterile instruments, open the thoracic cavity, and, from the heart's blood, inoculate a tube of pneumococcus broth and streak the other half of the blood agar plate which has been used for the peritoneal exudate (Fig. 176).

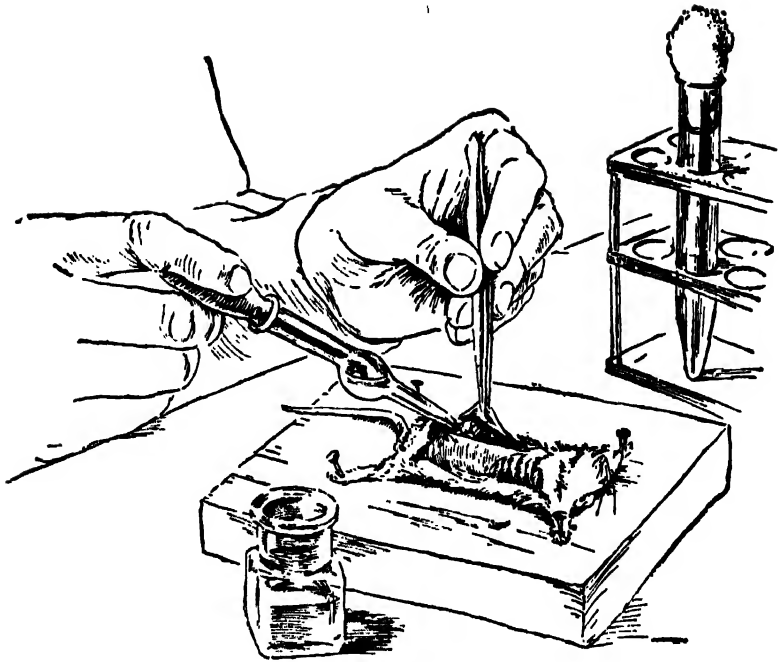


FIG. 175.—COLLECTING PERITONEAL WASHINGS  
(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

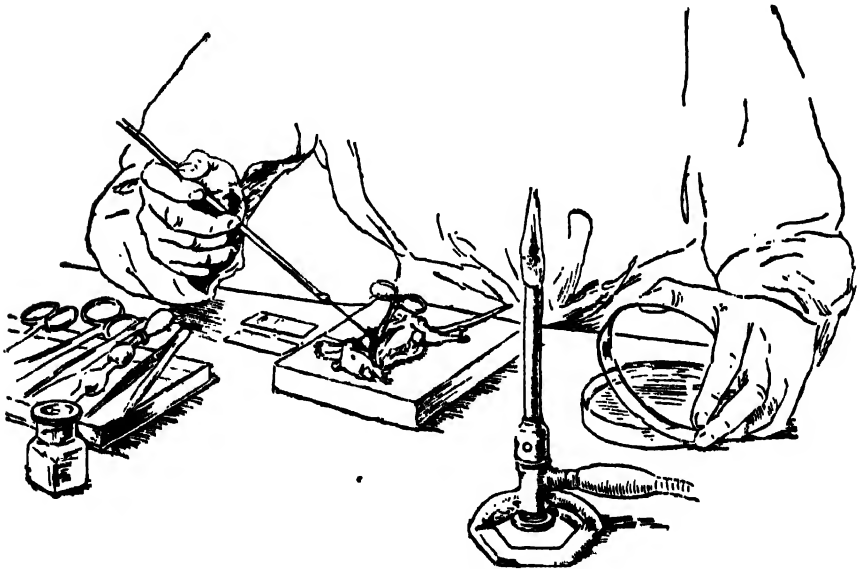


FIG. 176.—HEART BLOOD CULTURE  
(From Wadsworth, *Standard Methods*, The Williams and Wilkins Co., Baltimore.)

6. Centrifugalize the peritoneal washings at low speed for a few minutes; pour the supernatant suspension of organisms into a second centrifuge tube, and discard the sediment, which contains cellular debris from the peritoneum. Centrifugalize the suspension at high speed for from 15 to 20 minutes or until it is perfectly clear. Remove with a pipet the supernatant fluid.

7. Emulsify the sediment with saline solution, making a moderately heavy suspension (should equal at least an 18-hour broth culture of pneumococcus).

8. Place 0.2 cc. of this emulsion into each of 8 small test tubes.

9. Into the first 6 tubes place 0.2 cc. of the 6 pooled sera respectively. To the seventh tube add 0.2 cc. of saline solution and 2 drops of bile or 1 drop of a 10 per cent solution of sodium desoxycholate to determine if the emulsion contains chiefly bile-soluble pneumococci (control). To the eighth tube add 0.2 cc. of saline solution (control).

10. Mix well; place in a water bath at 37° C. for 1 hour.

11. Agglutination is indicated by a flocculent appearance in contrast to uniform cloudiness of the saline control, along with rapid settling of the agglutinated organisms leaving the fluid above clear. The bile control clears; if not, the suspension is probably not of pneumococci. Contaminating organisms, especially streptococci, may cause irregular agglutination reactions, but they are not dissolved by bile.

12. If a positive reaction occurs with a pooled serum, repeat the test with each of the separate or monovalent sera to determine the specific type of pneumococcus.

## METHODS FOR THE IDENTIFICATION OF GONOCOCCI

1. Materials submitted for examination for the gonococcus (*Neisseria gonorrhoeae*) are usually purulent secretions from the genital tract of both sexes, the vagina of children, sediment of urine, prostatic secretions obtained after massage, and the conjunctivae (especially of infants).

2. *Properly prepared cultures on suitable media have proven more valuable than smears in the diagnosis of gonococcal infections and as bacteriological guides in treatment and criteria of cure.*

3. The proper collection of material is very important, especially in relation to cultural examinations, because of the short viability time of gonococci. *Material collected on swabs should be promptly delivered to the laboratory for the preparation of smears and cultures.* The same is true of urine and vaginal washings of children. Otherwise, a delay in preparing cultures due to the transportation of materials, may result in falsely negative results due to the death of gonococci or the overgrowth of secondary organisms. No satisfactory method has yet been devised for meeting these conditions. For this purpose material for culture may be collected with sterile swabs, emulsified in 2 cc. of serum or ascitic hormone broth in 5 cc. vials, and shipped packed in ice or in thermos bottles. Cohn recommends a semi-solid medium because gonococci are less likely to undergo autolysis. The medium may be prepared of 20 gms. of Difco proteose peptone No. 3 and 7.5 gms. of plain agar in 1000 cc. of distilled water, autoclaved at 121° C. for 20 minutes and dispensed in sterile vials. Cultures of such material, however, give a high percentage of falsely negative results. For this reason it is advisable for physicians to prepare smears on glass slides and cultures on Petri plates or slants (former preferred) of "chocolate" blood agar, testicular hydrocele agar, cysteine mono-

hydrochloride serum hemoglobin agar or some other appropriate medium for shipment purposes.

4. Smears of material should be stained by the method of Gram. Fixation with 1 per cent solution of mercuric chloride is preferred to heat because pus cells are better preserved. Decolorization with acetone is recommended.

5. Typical gonococci are gram-negative. Duplicate smears stained with Löffler's methylene blue are advisable for morphology, but should not be relied upon alone for identification.

6. They are usually arranged in pairs, with adjacent sides flattened or slightly concave, resembling a pair of kidney beans. They are not encapsulated.

7. In the pre-acute stage before the exudate becomes profuse, the organisms may be extracellular, but become intracellular (Fig. 177) during the acute stage when the exudate is at its height. At this stage it is common to find many organisms gathered within 1 leukocyte while other cells in the immediate neighborhood have none. Later, when the infection becomes more chronic, the organisms become less numerous and are extracellular. In gonococcal conjunctivitis the organisms may occur in or upon epithelial cells.



FIG. 177.—GONOCOCCI WITHIN PUS CELLS

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

8. A *positive* report may be rendered when smears show large numbers of pus cells with gram-positive intracellular diplococci or typical cultures or both. Smears of urethral discharge from early cases and likewise from chronic cases (gleet) showing pus cells, many extracellular gram-negative diplococci and at least a few typical intracellular organisms, may be also reported as positive. Special care is required in reporting upon vaginal smears because other diplococci resembling gonococci are encountered more frequently than in the urethra; here cultures should be made, especially in medicolegal and treated cases.

A positive report may also be rendered in smears showing 50 per cent or more polymorphonuclear pus cells with many extracellular and occasional intracellular gram-negative diplococci of typical morphology.

9. A *suspicious* report may be rendered in cases with discharge when smears show many polymorphonuclear pus cells, but no intracellular diplococci.

10. A *negative* report may be rendered when smears show only a few polymorphonuclear pus cells with no suspicious intracellular diplococci and no clinical evidences of disease. In any positive, doubtful, or suspicious case, smears should be made once a week until a negative is obtained to be followed by 3 more well-made smears at intervals of about 3 days before one is prepared to render final report of negative.

11. As previously stated, properly prepared cultures are preferred to smears in the diagnosis of gonococcal infections. They are essential as bacteriological controls on treatment with the sulfonamide compounds or penicillin and as criteria of cure. Material may be collected on a sterile swab and the latter mixed well in 1 or 2 cc. of sterile ascites fluid or broth in a test tube. With this suspension prepare surface streak

plates. Many different media have been proposed. Those that are transparent are recommended, especially cysteine monohydrochloride serum hemoglobin agar and testicular hydrocele agar. Proteose or plain "chocolate" blood agar is usually quite satisfactory. In cases treated with the sulfonamide compounds it is advisable to add 5 mg. of para-aminobenzoic acid to each 100 cc. of medium.

12. After inoculation by the surface streak method, the plates should be incubated at 34° to 35° C. and preferably in an atmosphere carrying 10 per cent carbon dioxide.

13. On "chocolate" blood agar plates the colonies are usually quite characteristic if not too crowded, being fairly large, gray, translucent and often with irregular edges. They are definitely mucoid and when touched with a straight wire, the growth will "string up" like heavy gum from the rest of the colony. On crowded plates the colonies are tiny, without distinct character, and are difficult to recognize without the aid of the oxydase test.

14. The oxydase reagent is a 1 per cent solution of dimethyl-paraphenylene-diamine hydrochloride prepared 15 minutes beforehand. In conducting the test a few drops are placed on suspicious areas of the plate. Gonococcus colonies become pink, changing to red and finally to black. Organisms from pink colonies are usually viable and may be transferred to fresh media. The organisms of black colonies, however, are usually dead.

If the plate appears to be negative, the entire surface should be flooded with the reagent and examined after 15 to 30 minutes. This is usually necessary when plates are very crowded and only a few gonococci are present.

With only a few exceptions, the oxydase test is specific for the genus *Neisseria*. The only other oxydase positive organisms which are likely to occur with *Neisseria* are gram-negative bacilli. The reaction with these is usually atypical, the color developing more slowly and the final appearance being gray instead of black. Smears stained by the method of Gram must be studied very carefully since bipolar staining gram-negative bacilli closely resemble gonococci.

15. Prepare subcultures of the pink colonies on rubber-stoppered slants of 1 per cent glucose, lactose, sucrose, and maltose ascitic agar to which Andrade's indicator is added. Incubate at 35° C. for 48 to 72 hours. The gonococcus produces acid (no gas) only with glucose. Or prepare subcultures on the sugar agars without the indicator and after 2 or 3 days' incubation, allow a drop of 0.02 per cent aqueous solution of phenol red to flow over the surface (Bayne-Jones). A positive reaction is indicated by a change of color to yellow confined to the surface or to a thin superficial layer.

## METHODS FOR THE IDENTIFICATION OF MENINGOCOCCI

1. Materials submitted for examination for the meningococcus (*Neisseria intracellularis*) are usually spinal fluids, blood cultures and cultures of the nasopharynx.

2. In smears of spinal fluid, the meningococcus is usually arranged in pairs bearing a close resemblance to the gonococcus in morphology. When thoroughly decolorized they are gram-negative organisms but unless well decolorized may appear gram-positive with the possibility of being mistaken for the pneumococcus. They are not encapsulated.

3. Early in meningococcus meningitis the number of organisms present in smears

may be very few and largely extracellular, requiring careful examination. Later they become more numerous and largely intracellular (Fig. 178). As a general rule the finding of intracellular and extracellular gram-negative, coffee-bean shaped diplococci in spinal fluid justifies the immediate presumptive diagnosis of meningococcus meningitis, with the prompt institution of treatment.

4. Prepare surface streak cultures on plates of plain or "chocolate" blood agar. Serum or ascites agar plates may be employed. Incubate at 37° C. Since the meningococcus is carbon-dioxidophilic it is advisable to incubate the plates in an atmosphere carrying 10 per cent carbon dioxide. On ordinary blood agar plates the colonies are not particularly characteristic, closely resembling those of the coliform group. They are fairly large, gray, rather mucoid and nonhemolytic. On serum or ascites agar they are round, convex, bluish-gray, smooth and glistening with entire edges. Later they increase

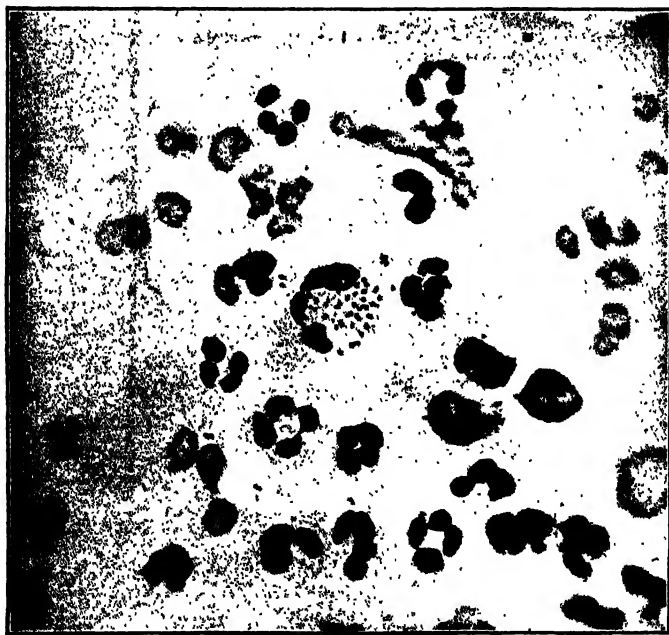


FIG. 178.—MENINGOCOCCUS IN SPINAL FLUID

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

in size, become more yellow and opaque and may show a granular center. In throat cultures for meningococci it is helpful to flood the plate for a few seconds with a 1 per cent aqueous solution of dimethyl-paraphenylene-diamine-hydrochloride. Colonies of meningococci, as well as those of all other gram-negative diplococci of the *Neisseria* group develop a pink, red and black color, but colonies of other organisms do not become discolored.

5. Prepare smears and stain by the Gram method. Gram-negative diplococci in cultures of spinal fluid are usually meningococci.

6. Test for fermentation by inoculating tubes of 1 per cent dextrose, maltose, levulose and sucrose agar to which Andrade's indicator has been added. Incubate for 48

to 72 hours. The meningococcus produces acid (no gas) with dextrose and maltose (Table 18).

7. Conduct an agglutination test for confirmatory purposes as follows in the case of gram-negative cocci recovered from cultures of spinal fluid, blood or nasopharyngeal cultures suspected of meningococci: (a) Subculture on slants of "chocolate" blood agar; (b) wash off 24-hour cultures with phenolized saline solution to secure a suitable suspension; (c) place 0.5 cc. of 1:10, 1:20 and 1:40 dilutions of group A antimeningococcus serum (types I and III usually causing epidemic meningitis) in a series of 3 small test tubes; (d) add 0.5 cc. of the antigen to each; (e) prepare a similar set of tubes employing group B antimeningococcus serum (types II and IV usually causing sporadic meningitis); (f) prepare a control carrying 0.5 cc. of saline solution and 0.5 cc. of antigen; (g) mix and place in a water bath at 37° C. for 1 to 2 hours; (h) positive agglutination indicates meningococcus.

**Rapid Method for the Detection of Meningococcus Carriers (Olitsky).—**

1. Material from the *nasopharynx* should be collected on a sterile cotton swab on a heavy wire, the last 2 cm. of which is bent at an angle of about 40 degrees, being careful to avoid possible contamination due to contact with secretions from the mouth or throat.

2. Inoculate warm plates of blood agar or serum agar and incubate at 37° C. for 24 to 48 hours.

3. Examine for colonies of meningococci. Prepare smears and stain by Gram method.

4. Transfer characteristic colonies of gram-negative diplococci to the following medium: Add 1 cc. of unheated, sterile, clear normal horse serum to 9.5 cc. of 1 per cent glucose broth and distribute 1 cc. amounts in small sterile tubes.

5. Incubate 12 to 24 hours and examine. The meningococcus produces a faintly turbid growth with slight sediment which emulsifies readily when shaken. Prepare smears and stain by Gram method.

*M. flavus*, *M. perflavus*, *M. sub-flavus*, *M. flavescens* and *M. pharyngis siccus* show gram-negative diplococci with firm agglutination by the normal horse serum with a clear supernatant fluid.

*M. catarrhalis* shows gram-negative diplococci with a dense turbidity often with a pellicle on the surface.

Staphylococci are gram-positive and produce a dense turbidity, an agglutinated sediment and often a pellicle.

Streptococci are gram-positive and produce a granular sediment with a clear or slightly turbid supernatant fluid.

*B. influenzae* fails to grow.

6. To such tubes as are suspicious of meningococci add 0.1 cc. of a 1:10 dilution of high-titer polyvalent antimeningococcus serum.

7. Incubate in a water bath (not an incubator) at 37 to 38° C. for 2 hours.

8. Prepare smears of tubes showing agglutination and stain by Gram method. Agglutinated masses of gram-negative diplococci are presumptive evidence of meningococcus.

9. Transplants may be made to slants of blood agar. Incubate 24 hours. Prepare smears and stain by Gram. Transplant cultures showing gram-negative diplococci to



slants of dextrose, maltose, levulose and sucrose agar to which Andrade's indicator has been added. Incubate 24 to 48 hours and examine for production of acid (gas is not produced):

TABLE 18

Production of Acid	Dextrose	Maltose	Levulose	Sucrose
<i>Meningococcus</i> .....	+	+	--	—
<i>M. catarrhalis</i> .....	—	—	—	—
<i>M. pharyngis siccus</i> .....	+	+	+	+
<i>M. perflavus</i> .....	+	+	+	+
<i>M. flavus</i> .....	+	+	+	—
<i>M. sub-flavus</i> .....	+	+	—	—
<i>M. flavescens</i> .....	—	—	—	—

**Serological Types of Meningococci.**—By agglutination and agglutinin absorption tests meningococci have been divided into Types I, II, III and IV. Types II and IV are closely related and often grouped together. In the United States the typing of meningococci is not usually requested since polyvalent sera are employed in treatment.

**Macroscopical Agglutination Test for the Differentiation of Meningococci.**—

1. Arrange 6 small test tubes.
2. Into Nos. 1, 2, 3 and 4 place 0.5 cc. of specific agglutinating sera for Types I, II, III and IV respectively; the dilutions to employ will depend upon the respective titers furnished with the sera.
3. Into No. 5 place 0.5 cc. of a 1:25 dilution of normal horse serum (control).
4. Into No. 6 place 0.5 cc. of saline solution (control).
5. Prepare a heavy uniform suspension of the meningococcus cultivated on blood or serum dextrose agar and place 0.5 cc. in each tube.
6. Mix well and place in a water bath at 55° C. for 3 hours. If desirable, they may be then placed in a refrigerator overnight before the readings are made.
7. If the organism is a meningococcus, agglutination should occur in the tube carrying the homologous serum. Inagglutinable strains are sometimes encountered.
8. If the organism is from the nasopharynx and agglutination occurs in all of the first 5 tubes (No. 6 is a control) the test is unsatisfactory as nonpathogenic diplococci found in this region are frequently strongly agglutinated by normal and immune horse sera in final dilutions of 1:50.

### METHODS FOR THE IDENTIFICATION OF *M. CATARRHALIS*

1. *M. catarrhalis* (*Neisseria catarrhalis*) occurs principally in the respiratory tract and may be encountered in cultures of the nose, throat and sputum. It is commonly regarded as but feebly pathogenic.
2. In smears of fresh material it occurs as a gram-negative diplococcus larger than the meningococcus and at times arranged in tetrads or small groups. In sputum the diplococci are shaped like coffee-beans, and may be both intra- and extracellular. They are not encapsulated.
3. The organism grows much more luxuriantly than the gonococcus and more freely than the meningococcus. On plain or blood agar the colonies at the end of 24

hours' incubation are convex, whitish-gray and glistening; after longer incubation they become more elevated, opaque, slightly brownish in the center with wave-like periphery, coherent, tenacious, difficult to emulsify and auto-agglutinable in saline solution. In broth there is usually no turbidity with a coarsely granular sediment difficult to break up by shaking; a pellicle may form. The organism is aerobic and will grow at 22° C. whereas the meningococcus and gonococcus require a higher temperature.

4. Sugar fermentation tests are usually required for final identification and differentiation from the meningococcus, *M. flavus*, *M. pharyngis siccus* and other gram negative diplococci occurring in the nasopharynx and sputum (Table 18). For this purpose inoculate rubber stoppered tubes of dextrose, maltose, levulose and sucrose agar to which the Andrade indicator has been added. Incubate 24 to 48 hours. *M. catarrhalis* does not produce acid or gas with any of these sugars.

### METHODS FOR THE IDENTIFICATION OF *B. ANTHRACIS*

1. The "malignant pustule" or anthrax of the skin produced by *B. anthracis* may be mistaken clinically for a simple furuncle or carbuncle.
2. It is usually encountered among workers in hides, hair and wools, but may be derived from shaving brushes or other articles.
3. Great care must be exercised in handling materials, cultures, etc. Be sure to thoroughly disinfect pus, glassware, etc., before discarding.
4. Septicemia may occur and blood cultures should be included routinely.
5. Anthrax of the bronchi may occur ("wool sorter's disease") in which case the bacillus occurs in the sputum.
6. Anthrax of the intestinal mucosa may also occur with the bacillus in the feces.
7. Prepare smears and cultures of the lesion and particularly of any blister fluid that may be obtained. Blood or plain glucose agar slants or plates may be employed. Avoid rough manipulation of the lesion which might spread the infection.
8. Stain smears by Gram method. The presence of scattered large bacilli with square ends is suspicious. Occasionally short chains are seen (Fig. 179). The bacilli are gram-positive if not decolorized too long. They may be encapsulated.
9. Incubate the cultures for 24 hours and examine. Growth is rapid. The colonies are dull, grayish-white, flat and spreading with irregular borders which when viewed under the lower power of the microscope have a Medusa-head appearance due to filamentous interlacing chains of bacilli. Examine smears stained by Gram method; spores are produced.
10. Examine for motility; *B. anthracis* is not motile.
11. Inoculate a Löffler blood serum slant and incubate 24 to 48 hours. *B. anthracis* produces very slight or no liquefaction.
12. If in doubt, inoculate a young guinea-pig subcutaneously or intraperitoneally with 1 cc. of a 24-hour broth culture or with a suspension in saline solution. *B. anthracis* usually produces a fatal septicemia in 12 hours to 2 to 3 days with the presence of organisms in the heart blood, spleen, liver and other organs. Prepare smears of blood and spleen; stain by Gram. Also prepare cultures on plain or blood agar. If an early diagnosis is desired, the suspected material may be inoculated subcutaneously into guinea-pigs, mice or rabbits without waiting for the isolation of pure culture.

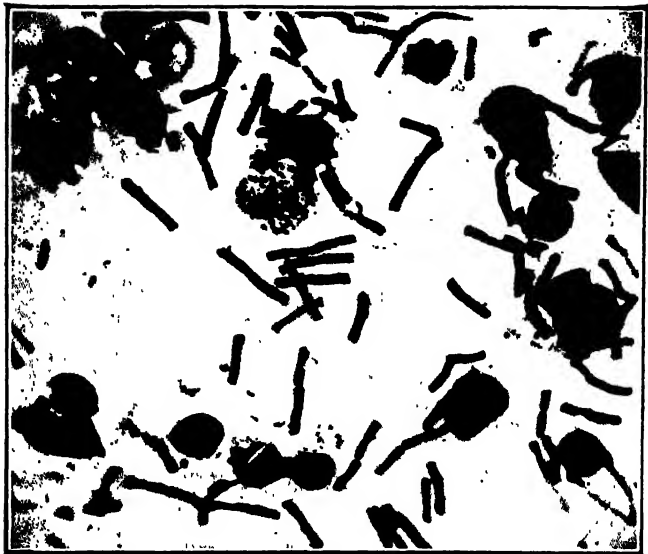


FIG. 179.—ANTHRAX BACILLI

Smear of spleen of animal dead of anthrax

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

13. *B. anthracis* must be particularly differentiated from *B. subtilis*; also from *B. mycoides* and *B. mesentericus*:

	Motility	Blood Serum	Milk	Virulence for Guinea-pigs
<i>B. anthracis</i> .....	Absent	No or slight digestion	Acid and coagulation	Virulent
<i>B. subtilis</i> .....	Present	Digestion	Alkaline	Nonvirulent
<i>B. mycoides</i> .....	Present	No digestion	Acid; no coagulation	Nonvirulent
<i>B. mesentericus</i> ....	Present	Slight digestion	Acid; no coagulation	Nonvirulent

14. If present in mixed culture, isolation is facilitated by heating a broth culture at 60° C. for 20 minutes and then plating.

15. A positive diagnosis of anthrax may be reported if the specimen contains a gram-positive, square-ended, chain-producing, spore-forming, nonmotile bacillus producing characteristic Medusa-head colonies on agar. Confirmatory tests are positive precipitation and virulence for young guinea-pigs, rabbits or mice.

**Precipitin Test.**—1. Prepare a suspension of a 24-hour agar slant culture in 5 cc. of saline solution.

2. Keep for 2 hours at room temperature and filter.

3. In a series of small test tubes place 1 cc. of undiluted, 1:10 and 1:20 dilutions of antianthrax serum.

4. Carefully overlay with 1 cc. of filtrate.

5. A positive reaction is indicated by a ring of precipitation within 15 minutes. Other aerobic, spore-bearing bacilli may give a reaction with undiluted serum.

**Ascoli Test.**—The Ascoli test for the detection of anthrax bacilli in meat may be conducted as follows:

1. Macerate the tissue with 5 to 10 parts of saline solution or 1:1000 acetic acid and boil for 15 minutes.
2. Centrifuge and filter through paper.
3. Place 0.5 cc. of antianthrax serum of good precipitating titer in a test tube and overlay with 0.5 cc. of clear filtrate.
4. Stand at room temperature for 15 minutes. A positive reaction is indicated by a white ring of precipitation.

**Blood Cultures.**—It is always advisable to culture 10 to 20 cc. of blood in 150 cc. of plain broth. The bacilli may be present and especially in some cases just before death. When positive they are usually present after an incubation of the culture for 24 to 48 hours.

**Isolation from Hair or Bristles.**—1. Rub up the suspected material in saline solution. Heat  $\frac{1}{2}$  at 80° C. for 30 minutes to kill nonspore-forming contaminants.

2. Centrifuge both portions. Prepare 4 agar plates of both sediments.
3. Inoculate mice and guinea-pigs with both.
4. Study colonies (special attention to deep ones).

## METHODS FOR THE IDENTIFICATION OF *B. SUBTILIS*

1. This organism, commonly known as the “hay bacillus,” is nonpathogenic, but is occasionally found as a saprophyte in old sinuses and infected wounds. It is also a common contaminant of culture media and may be mistaken for *B. anthracis*.



FIG. 180.—*BACILLUS SUBTILIS*

2. Typical colonies are large, dull, rough and hemolytic with very irregular edges. When held up to the light they present a typical ground-glass appearance. Colonies of other organisms of this group vary from the large, feathery, spreading growth of *B. mycoides* to small, smooth, shiny, compact colonies slightly resembling those of the coliform organisms. Sometimes the colonies show a definite green color or purplish iridescence.

3. *B. subtilis* is aerobic and, in broth, grows largely on the surface with a pellicle which later may drop to the bottom of the tube. It rapidly liquefies both gelatin and Löffler's blood serum medium.

4. The organism is gram-positive and occurs as straight rods (Fig. 180) in chains. Spores are found usually slightly nearer one pole than the other, but only in the chains.

5. It is actively motile whereas *B. anthracis* is nonmotile. For further differentiation see above under Methods for the Identification of *B. anthracis*.

### METHODS FOR THE IDENTIFICATION OF *B. DIPHTHERIAE*

1. Diphtheria is usually an infection of the upper respiratory tract (nose, fauces, or larynx) with *B. diphtheriae* (*Corynebacterium diphtheriae*). Laryngeal diphtheria is also known as "membranous croup." It is important to make smears and cultures (preferred) with much care as otherwise they may prove negative for the bacilli and misleading.

2. If smears of the exudate have been made, stain with Löffler's methylene blue. Cultures are much more reliable. In direct smears the bacilli are larger and atypical. Smears may be negative in cases giving positive cultures and should not be relied upon alone for bacteriological diagnosis.

3. The bacilli are gram-positive. Löffler's methylene blue is the best routine stain. There is no specific stain for differentiation from *B. hoffmannii* and diphtheroid bacilli. Neisser's stain may be used for bringing out more sharply the metachromatic granules.

4. By using sterile swabs physicians may make cultures on slants of Löffler's blood serum medium or on slants of potassium tellurite medium. The latter is preferred by many bacteriologists. Otherwise, cultures may be made in *Brahdys rapid culture tubes*. These are prepared by dipping sterile swabs in sterile horse serum, placing them in sterile test tubes and sterilizing in the autoclave at 121° C. for 30 minutes. After swabbing the throat or nose, the swab is replaced in the tube and stoppered. The latter should be incubated in a warm place (as in a vest pocket) until delivered to the laboratory. Diphtheria bacilli may be found in stained smears of the swab within 4 to 8 hours.

5. Cultures should be incubated at 35° to 37° C. for 18 to 24 hours. At higher temperatures the bacilli become smaller and atypical. Cultures may be examined as early as 8 to 12 hours, but the bacilli are larger, more solid and more difficult to recognize.

6. On Löffler's medium the colonies are small, circular, grayish or creamy white, convex and smooth. On potassium tellurite serum agar the colonies are opaque, raised, convex, shining and gunmetal in color with the centers usually darker in color than the periphery. On ordinary blood agar the colonies are usually small, white, opaque, with a very narrow zone of beta hemolysis. Occasionally nonhemolytic strains are observed.

7. The organism is aerobic. In broth a fine pellicle may develop on the surface with moderate turbidity and collection of fine granules on the sides of the tube, with a powdery deposit.

8. Prepare smears and stain with methylene blue. Be sure to pass the loop lightly over the entire surface of the slant in order not to miss isolated colonies. The smear will often show a pure or almost pure culture, owing to the fact that in the first 18 to 24 hours the bacilli tend to outgrow other organisms.

9. Diphtheria bacilli are slender, straight or slightly curved rods (Fig. 181) occurring in three principal morphological types, which Wesbrook and others have subdivided into many subvarieties:

- (a) *Granular or beaded* types, which are most typical and likely to be most virulent
- (b) *Segmented or barred* types, encountered less frequently, but likewise likely to be virulent
- (c) *Solid* types, which may be long or short and likely less virulent. These bacilli are readily mistaken for *B. hofmannii* and diphtheroid bacilli.



FIG. 181.—*DIPHTHERIA* BACILLI

10. Any of these, and especially the granular and segmented types, may have swollen ends producing the typical club-shaped bacilli. They are not encapsulated, do not form spores and are nonmotile.

(From Wood, *Clinical and Microscopical Diagnosis*, D. Appleton-Century Co., New York.)

11. It is usual and characteristic of diphtheria bacilli to lie at various angles to one another forming V or Y shapes, which when clumped give the appearance of Chinese letters. They do not occur in palisade formation or in chains as a general rule.

12. With experience *B. diphtheriae* may be readily identified by these means. But it is not always possible to safely differentiate the solid types from *B. hofmannii* and diphtheroid bacilli. Sugar fermentation and guinea-pig virulence tests are sometimes required.

13. The true diphtheria bacillus produces acid, but no gas in dextrose, maltose, and dextrin. Hiss serum or ascitic broth with 1 per cent of these sugars may be employed for the tests.

14. Three types of *B. diphtheriae* are recognized; *mitis* associated with mild diphtheria, *gravis* with severe fulminating diphtheria and an intermediate type. The

*gravis* type produces acid in starch serum broth with bromcresol purple converting the color of the indicator to yellow.

**Subcutaneous Test for Virulence.**—1. If the culture on a slant of Löffler's medium appears to be pure, wash it off with 10 cc. of sterile saline solution, emulsify and inject a 250 to 300 gram guinea-pig (not heavier) subcutaneously with 4 cc. in the median abdominal line. Inject a second pig with the same amount plus 1 cc. of antitoxin (100 to 500 units).

2. If the culture is not pure, first isolate the diphtheria bacilli by the "streak" method on plates of Löffler's blood serum, ascitic or blood agar.

3. Inoculate a tube of glucose broth with several different colonies. If one colony is used, there is danger of picking up a nonvirulent organism and securing a negative result even though virulent bacilli are actually present in the original culture on the slant.

4. Incubate at 37° C. for 48 hours, keeping the tube in a slanted position to give the culture as much oxygen as possible.

5. Examine for purity. Inject a 250 to 300 gram pig subcutaneously in the median abdominal line with 2 cc. of culture. Inject a second pig with the same amount plus 1 cc. of antitoxin (100 to 500 units).

6. Observe for at least 4 days. The control animal by either method should survive and show no edema at the site of inoculation.

7. Virulent bacilli by either method will kill the pigs within 4 days with marked hyperemia of the suprarenal glands. Or the pigs will be sick and show marked edema at the site of inoculation; this is also a positive result even though the pigs may not succumb in 4 days (they are likely to develop paralysis of the hind legs later on).

**Intracutaneous Tests for Virulence.**—1. The advantage of this method is that 2 or 3 cultures can be tested with one guinea-pig or rabbit. It is, however, less reliable than the subcutaneous tests.

2. Denude an area of abdominal skin by plucking out the hair for injection of each culture.

3. Emulsify a 24-hour growth of a Löffler slant in 10 cc. of sterile saline solution.

4. Inject 0.15 cc. intracutaneously.

5. Virulent bacilli produce a positive reaction of definite local inflammation in 24 hours which goes on to superficial necrosis in 48 to 72 hours.

6. A control animal may be inoculated in the same manner, but receiving an intraperitoneal injection of 100 to 500 units of antitoxin. It should show no lesions.

## METHODS FOR THE IDENTIFICATION OF *B. XEROSIS*

1. This is the special name given to the diphtheroid bacillus of the eye.

2. The bacillus may be found on the normal conjunctivae; it sometimes produces a low-grade chronic conjunctivitis.

3. Smears of conjunctival secretion stained by Löffler's methylene blue or Gram's method show solid bacilli of typical grouping and morphology.

4. Cultures on Löffler's blood serum or blood agar show luxuriant growths of large, solid, gram-positive bacilli of typical grouping; nonvirulent for guinea-pigs.

5. Cultures usually ferment some sugars in Hiss serum water medium, especially saccharose and dextrose but not dextrin.

**METHODS FOR THE IDENTIFICATION OF *B. HOFMANNII***

1. *B. hofmannii* is commonly found in the normal nose. It is now regarded as a separate species rather than a nonvirulent diphtheria bacillus. It is nonpathogenic.

2. The bacillus is shorter and thicker than *B. diphtheriae*; usually straight and slightly clubbed at one end (Fig. 182).

3. Stained with Löffler's methylene blue, the bacilli are solid but occasionally show an unstained transverse band through the middle which may give them a diplococcoid appearance. No polar bodies; no spores; not encapsulated; nonmotile, invariably gram-positive.

4. The organism grows more luxuriantly than *B. diphtheriae*. The colonies are larger, less transparent and whiter. In broth there is less turbidity and less tendency to form a pellicle.

5. Sugar fermentation tests are usually required for final identification. Inoculate a pure culture in Hiss serum water litmus medium carrying 1 per cent of dextrose, saccharose and dextrin. *B. hofmannii* produces no acid and no gas with these or other sugars.

6. The bacillus is nonvirulent for guinea-pigs.

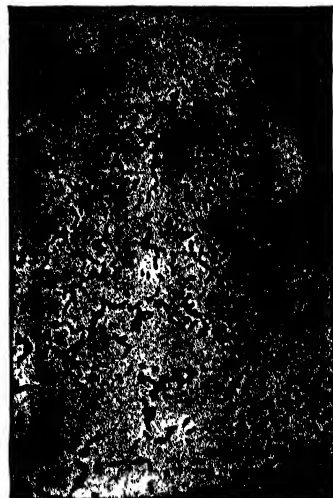


FIG. 182.—*BACILLUS HOFMANNII*

(From Wood, *Clinical and Microscopical Diagnosis*, D. Appleton-Century Co., New York.)

**METHODS FOR THE IDENTIFICATION OF DIPHTHEROID BACILLI**

1. Diphtheroid bacilli are widely distributed, commonly occurring in the normal nose and throat as well as on the skin, in lymphatic glands, ascitic fluid, etc.

2. They are commonly found in the pus of chronic infections and especially those involving bone (otitis media, wounds, fistulae, etc.) as secondary invaders.

3. They are nonvirulent, occurring in cultures of the nose and throat. They are readily mistaken for *B. diphtheriae* with unnecessary prolongation of quarantine following recovery from diphtheria.

4. The bacilli are usually long or short solid types, but often showing metachromatic granules.

5. These organisms grow on plain agar much more luxuriantly than *B. diphtheriae*. Surface colonies on blood agar are typically small, dull, flat, nonhemolytic with a slightly rough or stippled surface. They are usually quite hard and do not emulsify easily in broth. Some diphtheroid bacilli, however, produce larger, grayish-white, smooth colonies which may resemble *Staphylococcus albus*. Others are very tiny and, except for being pin-point in size, have no other distinguishing characteristics. There is nothing distinctive of deep colonies in blood agar plates since they are small and nonhemolytic.

6. The guinea-pig virulence test is the only reliable means for differentiation from *B. diphtheriae*.

7. They may produce acid with dextrose and dextrin in Hiss serum water litmus medium.



**METHODS FOR THE IDENTIFICATION OF *B. INFLUENZAE***

1. *B. influenzae* (*Hemophilus influenzae*) is not now usually regarded as the cause of influenza since it appears that a filtrable virus is the primary agent, but it is at least an important secondary organism and at times the primary cause of meningitis, pneumonia and arthritis.

2. It may be found in the nasopharynx of healthy persons.

3. The organism occurs as a very small, short, moderately thick rod, sometimes almost coccoid (Fig. 183) but is highly pleomorphic and may occur in long threads or filamentous forms, especially in spinal fluid. Bipolar granules are frequently seen.

4. *B. influenzae* is gram-negative; not encapsulated; does not form spores; is nonmotile.

5. It is best stained with dilute carbolfuchsin or Giemsa's stain.

6. Smears of spinal fluid (usually prepared of sediment after centrifuging) are of great value in diagnosis but direct smears of the nasopharynx are not worth while.

7. Being a strict parasite it requires for cultivation the presence of accessory substances or "growth factors," of which two, X and V, have been distinguished. The X factor, which is thermostable, is associated with hematin and with less well-defined substances present in fruits, potato and other vegetables containing iron. The V factor, which is thermolabile, is found in blood, potato and yeast. It is a coenzyme I or II composed among other things of nicotinic acid amide of vitamin B complex.

8. Satisfactory media are "chocolate agar," fresh blood agar (human or rabbit blood preferred) and Avery's sodium oleate agar (pH 7.2).

9. Sputum should be obtained from the lower passages and washed in sterile water. Select one or more solid particles and streak on plates. In the case of spinal fluid culture at least 1 cc. or 0.5 cc. of sediment. Swabs of the nasopharynx may be streaked on plates.

10. Cultivate aerobically at 37° C. Surface colonies on plain blood agar plates are quite characteristic and this, along with a pronounced odor, makes their recognition fairly easy. The colonies are small, colorless, almost transparent, nonhemolytic and have a characteristic dew-drop appearance. When the plate is held up to the light and the colonies observed from the under side, they are very striking, appearing as shining points of light. Deep colonies are small and nonhemolytic with no distinct characteristics. Upon removal of the cover of a plate a characteristic odor is noted.

11. If the culture is contaminated with other organisms, especially *Staphylococcus aureus*, the colonies are considerably larger, more opaque, of a grayish white color,

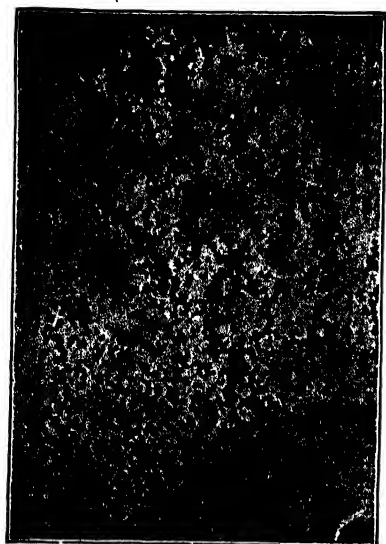


FIG. 183.—*BACILLUS INFLUENZAE*

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

and develop more luxuriantly in the neighborhood of the foreign colony, a phenomenon termed the "*satellite phenomenon*".

12. Prepare smears and stain by Gram method. Transplant colonies to slants of plain and "chocolate agar". *B. influenzae* will not grow on the former.

13. It is a difficult organism to identify until considerable experience has been gained. In spinal fluid, however, it is readily identified by these characteristics. Most strains produce acid (no gas) with levulose, galactose and dextrose, but not with lactose or mannitol.

14. The meningeal type of *B. influenzae* is agglutinated by meningeal-type anti-serum, the test being conducted in the same manner as for the identification of meningococci.

15. Influenzae bacilli belonging to type B show capsular swelling ("quellung reaction") when treated with type B antiserum (Lederle Laboratories). The test is conducted with cultures, spinal fluid or nasopharyngeal mucus in the same manner as the Neufeld test for typing pneumococci.

## METHODS FOR THE IDENTIFICATION OF *B. PERTUSSIS*

1. It is difficult to isolate *B. pertussis* (*Hemophilus pertussis*) of Bordet-Gengou, even under the most favorable circumstances, because it grows slowly.

2. Good results are secured by culturing the thick viscid pellets of sputum obtained during the early or catarrhal stage as the secretions of the nose usually give negative results. The "cough plate" method devised by Chievitz and Meyer is preferred.

3. The examination of stained smears of sputum is hardly worth while as the bacillus cannot be differentiated from *B. influenza* by morphology alone.

4. *B. pertussis* requires neither the X nor V factors for its growth and the best medium is the glycerin-potato-blood agar of Bordet-Gengou or modifications of it (page 352), with a pH of about 5.0 as it inhibits the growth of *B. influenza*. Mueller believes that better results are obtained by leaving the medium unadjusted which gives a pH of 6.1 to 5.8.

5. Pellets of thick tenacious mucus should be taken up on a platinum loop, washed 2 or 3 times in sterile saline and streaked on plates.

6. With infants and young children from whom sputum may not be obtainable, open plates of medium may be held 5 or 6 inches in front of the mouth during a paroxysm of coughing or forced expiration ("cough plates").

7. Incubate 48 to 72 hours. The colonies are characteristic being small, dome-shaped, moist and pearly-white resembling beads of mercury surrounded by a narrow darkened zone of hemolysis.



FIG. 184.—*BACILLUS PERTUSSIS*

Organisms from forty-eight-hour culture on Bordet-Gengou medium. (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

8. The colonies do not show the "satellite phenomenon" which is a valuable aid in differentiating them from colonies of *B. influenzae*.

9. Prepare smears and stain by the method of Gram. Best results are obtained with phenol-toluidine blue (Bordet-Gengou) or phenol-methylene blue. It is gram-negative and does not form spores.

10. The bacillus occurs as small ovoid rods (Fig. 184) sometimes bipolar and resembling *B. influenzae*. The forms seen in cultures are often larger than those seen in smears of mucus and filaments are sometimes produced.

11. *B. pertussis* does not ferment any carbohydrate, does not reduce nitrates and forms no indol.

12. Highly virulent pertussis bacilli (phase I) are agglutinated by phase I anti-serum.



FIG. 185.—CONJUNCTIVAL SMEAR SHOWING KOCH-WEEKS BACILLI

P, intracellular bacilli.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

### METHODS FOR THE IDENTIFICATION OF THE KOCH-WEEKS BACILLUS

1. The Koch-Weeks bacillus (*Hemophilus conjunctivitis*) causes acute contagious conjunctivitis commonly known as "pink eye".

2. Diagnosis is best made by the examination of smears of conjunctival secretions stained by the method of Gram.

3. The organism occurs as a small gram-negative bacillus slightly larger than *B. influenzae*. It is usually intracellular but also extracellular (Fig. 185). In acute cases the bacilli are present in large numbers but are so small that they may be overlooked. They tend to occur in shoals near pus cells. Smears stained with dilute carbolfuchsin or phenol toluidine blue are sometimes to be preferred.

4. As a general rule such findings are sufficient for diagnosis.

5. If cultures are desired, inoculate plates of fresh blood agar as both growth factors, X and V, are required. Ascitic-glycerin agar is also suitable. Incubate for 48 hours. Examine

for small, dewdrop colonies of gram-negative bacilli. The bacillus does not ferment the sugars.

### METHODS FOR THE IDENTIFICATION OF THE MORAX-AXENFELD BACILLUS

1. The Morax-Axenfeld bacillus (*Hemophilus lacunatus*) produces a type of sub-acute or chronic angular conjunctivitis.

2. Prepare smears of the conjunctival secretions and stain by the method of Gram.

3. The organisms appear as short, thick bacilli, usually in the form of 2 bacilli placed end to end, but infrequently singly or in short chains. Their ends are distinctly rounded, their centers slightly bulging, giving the bacillus an ovoid form (Fig. 186). They are gram-negative; are not encapsulated, and do not form spores.

4. Such findings are usually sufficient for diagnosis.

5. If cultures are made, use alkaline agar enriched with serum, blood or ascitic fluid. The organism does not require the X or V factors. Incubate at 37° C. for 48 hours. The colonies are gray and at first almost invisible. On Löffler's blood serum colonies appear as small indentations or erosions due to liquefaction of the medium from which the name "lacunatus" is derived.

6. The bacillus produces acid but no gas with dextrose and mannitol but not with maltose or lactose.

#### METHODS FOR THE IDENTIFICATION OF THE ZUR NEDDEN BACILLUS

1. This bacillus is believed to sometimes produce corneal ulcers.

2. It is very small, often slightly curved and generally occurs singly as it does not form chains.

3. Prepare smears and stain by method of Gram. It is gram-negative, often staining poorly at the ends. It is not encapsulated.

4. It grows readily on ordinary culture media. Upon plain or blood agar the colonies are transparent, rounded, raised, granular and slightly fluorescent with a tendency to confluence. Gelatin is not liquefied; milk is coagulated; indol is not produced. On dextrose culture media it will produce acid but not gas.



FIG. 186.—MORAX-AXENFELD DIPLOBACILLUS

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

#### METHODS FOR THE IDENTIFICATION OF THE BACILLUS DUCREYI

1. The bacillus of Ducrey (*Hemophilus ducreyii*) is regarded as the cause of chancroidal infection ("soft chancre").

2. The best method of examination consists in aspirating an unopened bubo and preparing smears and cultures of the pus. When buboes are not present, material may be scraped from the base of the ulcer or from beneath its overhanging edge with a stiff wire.

3. Stain the smears by the method of Gram.

4. The bacillus is difficult to cultivate; the method of Teague and Deibert is recommended: (1) Bleed a rabbit aseptically from the heart and distribute 1 cc. amounts in small sterile test tubes. Allow to clot, heat for 5 minutes at 55° C. and keep in refrigerator. (2) With sterile stiff iron wire bent at one end, secure pus by gently rubbing over the base of the ulcer or under its undermined edge and then pick up a bit of pus from the dressing. (3) Transfer to a tube of the clotted blood and distribute in the serum. (4) Incubate at 37° C. for 24 hours. (5) Stir the serum with a platinum loop and prepare a smear; stain by the method of Gram.

5. The presence of extremely small gram-negative bacilli with no capsules and no

spores are usually sufficient for diagnosis. They have a tendency to occur in short chains and in parallel rows. In smears of pus they are often intracellular. They stain more deeply at the poles.

6. If cultures are made use plates of 1 part of fresh sterile blood with 2 parts of agar inoculated by the surface streak method. Incubate at 37° C. for 48 hours and examine for very small, transparent, gray, firm and finely granular colonies. Prepare smears and stain by the Gram method.

### METHODS FOR THE IDENTIFICATION OF BACTERIUM TULARENSE

1. *Bacterium tularense* (*Pasteurella tularensis*) produces a disease of rodents transmissible to man with the production of four chief clinical types of disease: (a) ulceroglandular, due to infection of the skin and later of the regional lymphatic glands; (b) oculoglandular, due to infection of the conjunctivae and later of the lymphatic glands; (c) glandular, with no primary lesion; and (d) typhoid, with no primary or glandular lesions.

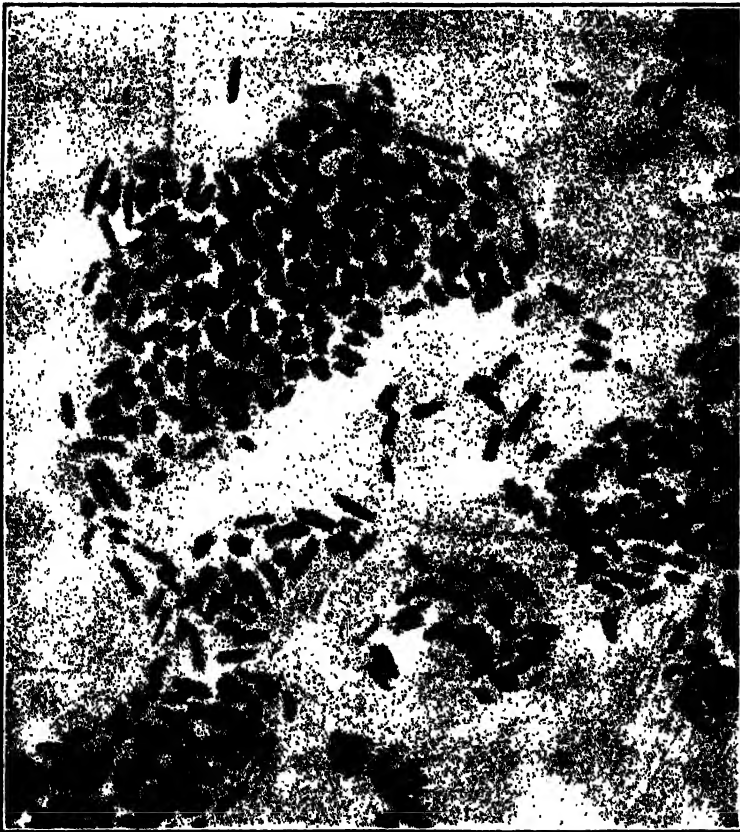


FIG. 187.—BACTERIUM TULARENSE FROM CULTURE ON GLUCOSE CYSTINE AGAR, SHOWING COCCOID AND BACILLARY FORMS IN THE SAME FIELD. (Army Medical Museum. Courtesy Edward Francis. U.S.P.H.S.) Approx.  $\times 5000$ .

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

2. If secretions are available prepare smears on slides and stain by the Gram method. It is also advisable to make a darkfield examination for *Spirochaeta pallida* to rule out the possibility of syphilis.

3. Inoculate tubes or plates of glucose-cystine agar or blood-cystine agar by rubbing secretion, pus, blood, or better still, a piece of infected tissue over the surface of the medium. Make a blood culture.

4. Incubate at 37° C. for 3 to 5 days and examine for minute, buttery, smooth, easily emulsified, grayish-white colonies. If negative, incubate and study for at least 2 or 3 weeks longer. Prepare smears and stain by Gram method.

5. The bacilli are small, gram-negative, nonmotile, nonspore forming and usually occur singly (Fig. 187). They are pleomorphic: bacillary, coccoid and frequently bipolar. A clear area resembling a capsule often surrounds the bacilli in animal lesions or when mixed with serum. Carbol-fuchsin and gentian violet are the best stains.

6. The bacterium produces acid but no gas with glucose, levulose, mannose and glycerin.

**Animal Inoculation Test.**—1. Inject a guinea-pig subcutaneously with material from glands, ulcers or blood. 2. Shave or pluck the abdominal skin of a second animal; produce and inoculate abrasions. 3. As a general rule both animals will die in 5 to 10 days if *B. tularensis* is present with hemorrhagic edema but no pus at the site of inoculation; the lymphatic glands are enlarged with dry, yellowish, caseous material; the spleen is enlarged and dark in color; the liver contains many discrete, white, caseous granules. Prepare smears and cultures.

## METHODS FOR THE IDENTIFICATION OF *B. PESTIS*

1. *B. pestis* (*Pasteurella pestis*) produces a disease of rodents transmissible to man. In the latter two varieties occur: (a) *bubonic plague* due to infection of lymphatic glands and (b) *pneumonic plague* due to infection of the lungs.

2. In the bubonic type prepare smears and cultures of pus aspirated or taken from the glands. If these are small, hard and difficult to aspirate, excise a gland. In the pneumonic type prepare smears and cultures of the sputum. A blood culture is advisable in all cases. From cadavers secure material from glands, spleen and lung.

3. Stain smears by the method of Gram; also with methylene blue or dilute carbol-fuchsin.

4. Inoculate plates of blood agar, glycerol agar or 3 per cent sodium chloride agar by the surface streak method. For blood cultures use nutrient broth.

5. Incubate at 30 to 35° C. for 48 hours and examine.

6. The bacilli are short and thick with rounded ends and convex sides, occurring singly or in pairs and at times in short chains or small groups (Fig. 188). They are highly pleomorphic with marked variations in size and staining. They are gram-negative and bipolar; nonmotile; nonsporulating. In the tissues and in serum broth, capsules are sometimes present.

7. The colonies are small, round, glistening, transparent, colorless, purely granular and entire or with slightly undulating edges. No hemolysis on blood agar. No liquefaction of Löffler's blood serum medium.

8. In broth there is moderate growth with a delicate pellicle from which thread-like growths (stalactites) hang down in the medium.

9. Produces acid, but no gas in media carrying dextrose, maltose, mannitol and salicin. Litmus milk is acidulated but not coagulated. Gelatin is not liquefied.

10. Conduct agglutination test with high-titer serum and an antigen of bacilli for confirmation.



FIG. 188.—*BACILLUS PESTIS*  
(After Mallory and Wright.)  
(From Zinsser and Bayne-Jones, *Text-book of Bacteriology*, D. Appleton-Century Co., New York.)

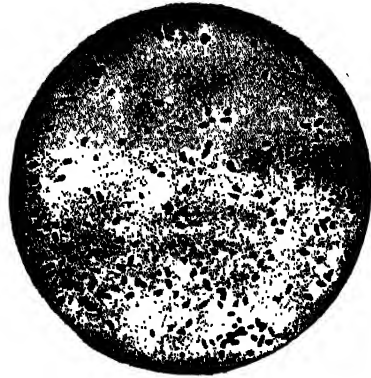


FIG. 189.—*BACILLUS PYOCYANEUS*  
(From Park, Williams and Krumwiede, *Pathogenic Microorganisms*, Lea and Febiger, Philadelphia.)

**Animal Inoculation Test.**—1. Use guinea-pigs or mice.

2. Several hours before inoculation, dip the animals in an antiseptic solution to kill all ectoparasites and then place them in greased glass jars covered with fine mesh top to prevent the escape of any infected parasites.

3. Inoculate subcutaneously with small amounts of original material or with a few loopsful of suspected culture.

4. If *B. pestis* is present, the animals will die in 2 to 4 days with the characteristic lesions in the spleen, liver, etc.

5. Prepare smears and cultures.

**Diagnosis of Plague in Rodents.**—1. If to be sent to a distant laboratory place the specimen in a tightly sealed container, which is packed in a second container to avoid breakage and ship by express as federal laws prohibit shipment by mail. Decomposition may be avoided by surrounding the specimen container with solid carbon dioxide (dry ice).

2. Wear rubber gloves and long sleeved gowns.

3. Dip the animal in antiseptic solution to kill fleas and other parasites.

4. Conduct an autopsy for macroscopic lesions, although occasionally the disease may occur without recognizable lesions.

5. Examine lymphatic glands which are enlarged, congested, hemorrhagic and later-necrotic.

6. Examine the liver for mottling, punctate hemorrhages and pinpoint yellowish spots of fat necrosis.

7. Examine the spleen for enlargement with small, discrete or confluent granules on the surface.

8. Examine the pleural and peritoneal cavities for excess of fluid.

9. Prepare cultures. Examine sections of tissues stained for the bacilli.

10. Tularemia of rats resembles plague so closely that it is necessary to differentiate culturally. The *Corynebacterium pseudotuberculosis* (*Pasteurella pseudotuberculosis*) also produces similar lesions but differs from *B. pestis* in being motile in young broth cultures at 22° C. and by fermenting saccharose with production of alkalinity in milk. The lesions produced by *Trypanosoma lewisii* can be differentiated by finding this parasite in blood smears.

### METHODS FOR THE IDENTIFICATION OF *B. PYOCYANEUS*

1. *B. pyocyaneus* (*Pseudomonas aeruginosa*) is widely distributed in nature and is ordinarily of low pathogenicity. It is particularly likely to occur as a secondary invader in chronic suppurations and especially those involving bone (chronic otitis media; suppurative mastoiditis; osteomyelitis; infected wounds, sinuses, etc.). It may, however, produce primary infections and especially in poorly nourished children (acute otitis media; diarrhea and gastro-enteritis, etc.). It occasionally produces septicemia.

2. In smears and cultures it occurs as a short, slender (Fig. 189), gram-negative bacillus, ordinarily single but sometimes in chains of 4 to 6 elements and occasionally growing out into long filaments and twisted spirals. Sometimes granules are present and the organism may be mistaken for the diphtheria bacillus which, however, is gram-positive.

3. It stains readily; is noncapsulated, actively motile and nonsporulating.

4. *B. pyocyaneus* grows luxuriantly on ordinary media at 37° C. On blood agar plates the surface colonies may be recognized with a fair degree of accuracy by their appearance and characteristic odor. They are usually large, greenish-gray, extremely irregular in shape and present a stippled appearance. They may spread slightly in an irregular zone around the colony. They may appear iridescent by transmitted light and have a metallic sheen by reflected light. The typical green pigment may or may not be apparent on blood agar. On desoxycholate agar the colonies may be small, translucent and colorless. After 48 hours' incubation, however, they are readily recognized by their dark green-brown centers. On all solid media the colonies have a characteristic gummy consistency.

5. The organism in aerobic cultures produces characteristic pigments which diffuse into the medium. One of these is *fluorescein*, which is greenish-yellow or green, soluble in water but insoluble in chloroform. The second is blue-green or *pyocyanine*, soluble in both water and chloroform. It grows readily in broth and especially on the surface with the formation of a pellicle. Occasionally the color is apparent, but more often, due to oxygen requirements, it is necessary to shake the tube vigorously to produce a noticeable pigment.

6. It rapidly digests Löffler's serum medium and gelatin; produces acid, but no gas, in dextrose; produces no indol; does not reduce nitrates.

### METHODS FOR THE IDENTIFICATION OF *B. MUCOSUS CAPSULATUS*

1. *B. mucosus capsulatus* (*Klebsiella pneumoniae*) or the Friedländer bacillus is usually found in the respiratory tract associated with or producing chronic sinusitis, otitis media and mastoiditis, bronchitis, bronchiectasis, pneumonia and pleuritis, and



occasionally pericarditis, conjunctivitis, meningitis and septicemia. It is also important in relation to infections of the urinary tract.

2. A heavily encapsulated gram-positive bacillus isolated from the sputum in pneumonia, or the spinal fluid in meningitis, is usually regarded as a Friedländer's bacillus. The same organism isolated from a urine culture might be wrongly classified as *aerobacter aerogenes*. Therefore, designation of an organism as Friedländer's bacillus depends primarily on the source of the culture.

3. In smears of pus and cultures it occurs as short, plump bacilli or diplobacilli; singly and occasionally in short chains (Fig. 190).

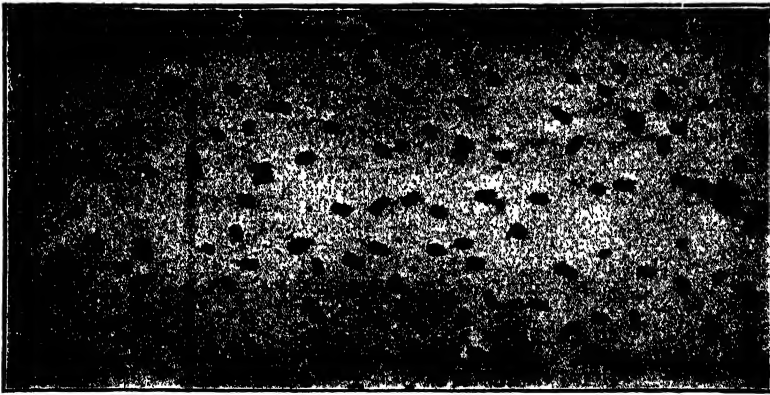


FIG. 190.—*BACILLUS MUCOSUS-CAPSULATUS*

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

4. The organism stains readily and is gram-negative; *encapsulated* (Fig. 191); nonmotile; nonsporulating.

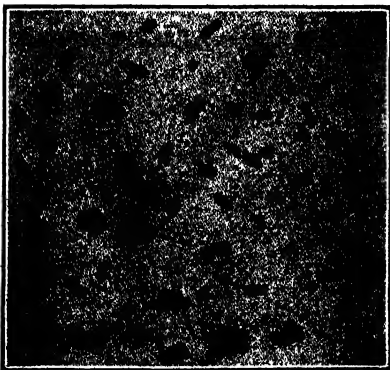


FIG. 191.—*BACILLUS MUCOSUS CAPSULATUS*

(From Ford, *Textbook of Bacteriology*, W. B. Saunders Co., Philadelphia.)

5. It grows well on ordinary media at 37° C. Surface colonies on plain blood agar are very large, gray, mucoid, heaped-up and nonhemolytic with a marked tendency to become confluent. In consistency they are very soft and mucoid, and when touched with an inoculating needle, the growth "strings up" in long threads, leaving the surface of the colony intact.

6. It grows well in broth with a pellicle and, after several days, a marked viscosity.

7. As a general rule the organism is readily identified by these characteristics. Litmus milk is acidified and sometimes coagulated. Acid and gas are produced in dextrose, levulose, galactose, lactose and sucrose; gelatin is not liquefied.

8. By serological tests the smooth type encapsulated bacilli are divisible into types A, B, C and a heterogeneous Group X. These types are biochemically heterologous and do not always ferment lactose with the production of gas. The bacilli are placed in

the coliform group because of morphologic and biochemical resemblances to *B. coli* (See Table 21 on page 469).

9. At the present time serum is available (Lederle Laboratories) for the identification of types A and B by the Neufeld "quellung" method. The technic is the same as that described for the typing of pneumococci by the Neufeld "quellung" reaction.

### METHODS FOR THE IDENTIFICATION OF THE BACILLUS RHINOSCLEROMATIS

1. The bacillus of rhinoscleroma (*Klebsiella rhinoscleromatis*) produces a chronic granuloma of the nose, mouth, pharynx and larynx; it is rare in the United States.

2. It is secured by cultures of the lesion removed by biopsy and cultured in broth or on plain or blood agar. It is also found in the "Mikulicz cells" of stained sections of tissue (Fig. 192).

3. The bacillus is a plump, short rod, with rounded ends, morphologically and culturally very similar to the *B. mucosus capsulatus* of Friedländer. It is nonmotile, gram-negative and encapsulated.

4. It differs from the Friedländer bacillus in producing no gas in dextrose broth, no acid in lactose broth, and not coagulating milk.



### METHODS FOR THE IDENTIFICATION OF *B. OZENAE*

1. The etiology of ozena or atrophic rhinitis is uncertain. *B. ozena* (*Klebsiella ozaenae*) is of doubtful relation to the disease.

2. The bacillus is quite similar to the Friedländer bacillus in being gram-negative encapsulated rods but cultures on solid media are said to be more watery and in gelatin stab cultures *B. ozena* spreads out over the surface while the Friedländer bacillus forms the characteristic "nail head" growth.

3. According to Page, freshly isolated strains of *B. ozena* show a characteristic delay in the production of gas with saccharose and sorbose (5 days) and a longer delay in lactose (8 days).

4. Another bacterium supposed to have an etiological relationship to ozena is the bacillus of Perez (*Cocco-bacillus foetidus ozaenae*) which is gram-negative, nonmotile and noncapsulated. It grows well on ordinary media, does not liquefy gelatin, produces indol and a characteristic fetid odor in cultures.

FIG. 192.—BACILLUS OF RHINOSCLEROMA

Section of tissue showing the microorganisms within Mikulicz cells. (After Fränkel and Pfeiffer.) (From Zinsser and Bayne-Zones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

**METHODS FOR THE IDENTIFICATION OF *B. MALLEI***

1. *Bacillus mallei* (*Pfeifferella mallei*), which is the cause of glanders in horses, mules and occasionally in other lower animals, sometimes produces the disease in human beings whose occupation brings them in contact with diseased animals.

2. Infection takes place by entrance through the broken skin and the mucosa of the mouth or nose. In man infection usually occurs through the skin.

3. Bacteriological diagnosis is usually difficult. Pus from the skin lesions may be used but better results are secured with freshly excised lesions of the skin or portions of submaxillary lymph glands. These should be finely divided with sand in a sterile mortar and planted on plates of 3 per cent glycerol-agar (pH 7.6), glycerol-potato medium, and in 3 per cent glycerol-broth (pH 7.6).

4. Direct smears may be prepared and stained with carbolfuchsin or Löffler's methylene blue but are hardly worth while since the bacillus is not readily recognized by morphological characteristics alone when mixed with other bacteria.

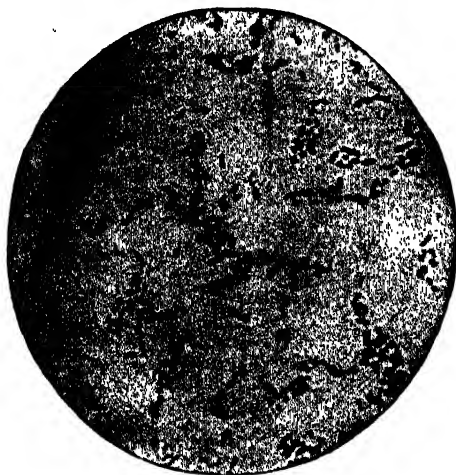


FIG. 193.—GLANDERS BACILLUS

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

5. Incubate the cultures for 48 to 72 hours as the bacillus at first may grow rather slowly. On glycerol-agar the colonies are whitish or yellowish and usually round. On potato the growth is more characteristic being yellowish, semitransparent and like drops of honey, gradually becoming brownish or amber colored and tenacious. The medium may become green or greenish-brown.

6. Pick off suspicious colonies and transfer to agar slants. Stain smears by Gram method and with carbolfuchsin.

7. The bacillus is gram-negative, slender, nonmotile, nonsporulating and nonencapsulated (Fig. 193). In cultures it is highly pleomorphic, varying greatly in length and width, often showing irregular bizarre forms, and occasionally long filaments with false branching. They

stain rather faintly and are sometimes bipolar.

8. Culture can be definitely identified by agglutination test with immune serum, although freshly isolated strains are not easily agglutinated. In conducting this test, heat several 48-hour glycerin-agar slant cultures at 60° C. for 2 hours and suspend the growths in saline solution carrying 0.3 per cent tricresol. Shake well for a homogeneous suspension and filter if necessary through soft paper.

Set up agglutination tests with 0.5 cc. of varying dilutions of a known immune serum and add 0.5 cc. of the bacterial suspension. Include saline controls and preferably a set of controls employing normal serum.

Incubate at 37° C. for 24 hours and read the reactions.

**Inoculation of Animals (Straus Test).**—1. Inject intraperitoneally a male guinea-pig with a small amount of suspected material.

2. If *B. mallei* is present, an orchitis will develop in 3 to 4 days in about 60 to 70 per cent of cases; in acute glanders positive results are much more likely than in chronic glanders since the bacilli may become avirulent. Similar lesions may be produced by *C. pseudotuberculosis*, *L. whitmori* and even by tuberculosis or diphtheria bacilli.

3. As soon as the orchitis is well developed, destroy the animal and culture the testes as just described. If the animal is allowed to live, the testes will abscess and discharge. Lesions will also develop in the liver, spleen, pancreas, lungs, etc.

### METHODS FOR THE IDENTIFICATION OF THE BRUCELLA

1. *Brucella melitensis* infects goats; *Brucella abortus* infects cows and *Brucella suis* infects hogs. Man may become infected through the ingestion of milk from infected goats and cows or by the flesh of these animals.

2. The three organisms are closely related. In man, laboratory diagnosis is attempted by cultures of the blood, urine and feces and by agglutination and complement fixation tests with serum.

3. Blood cultures are prepared with 10 cc. or more of blood in 250 to 500 cc. of infusion broth or liver infusion broth. Incubate at 37° C. and observe for at least 3 weeks, making frequent transfers to duplicate sets of plates of glycerin-agar, "chocolate" blood agar and liver infusion agar. Incubate one set of plates in a jar with 10 per cent CO<sub>2</sub>. Examine the plates daily. *Br. abortus* grows fairly well in CO<sub>2</sub> while *Br. melitensis* and *Br. suis* grow very poorly.

4. Collect urine aseptically by catheterization and centrifuge. Inoculate the 3 media with sediment by the surface streak method and incubate as above. The milk of goats and cows may be cultured in the same manner using both the cream and sediment.

5. The colonies are small, round, convex, smooth, glistening and almost colorless. Prepare smears and stain by Gram method.

6. The organisms occur as short straight rods (almost coccil), singly, in pairs end to end, in small groups and occasionally in short chains. They are gram-negative and may show polar staining. They are noncapsulated and nonmotile.

7. Transfer colonies to "chocolate" blood agar. Incubate at 37° C. and examine for purity. Identify the species by the method of Huddleson: prepare plates of beef liver infusion agar (pH 6.6) with (1) 1:50,000 thionin; (2) 1:25,000 basic fuchsin and (3) 1:100,000 pyronin. Inoculate by the surface streak method and incubate 24 to 48 hours; examine:

TABLE 20

Species	CO <sub>2</sub> required for isolation	H <sub>2</sub> S	Growth in Presence of		
			Basic fuchsin	Thionin	Pyronin
<i>Br. abortus</i> . . . . .	+	+++	+++	—	+++
<i>Br. melitensis</i> . . . .	—	±	+++	+++	+
<i>Br. suis</i> . . . . .	—	+++	—	+++	—

8. Test for the fermentation of dextrose. *Br. melitensis* and *Br. suis* produce more acid than *Br. abortus*.

9. In a medium containing organic sulphur, *Br. abortus* and *Br. suis* liberate hydrogen sulphide while *Br. melitensis* does not.

10. Final identification may require macroscopic agglutination tests with antisera for *Br. melitensis*, *Br. abortus* and *Br. suis*; the final dilutions to employ depends upon the titers of the respective sera. Owing to the difficulty of bacteriological diagnosis, agglutination and complement fixation tests with patient's serum are preferred. When agglutination tests are conducted, it is advisable to conduct duplicate tests with *B. typhosus* and *B. tularensis* at the same time because of the difficulties sometimes arising in differential diagnosis.

**Animal Inoculation Test.**—The organisms may be isolated from infected tissues, blood, urine or milk by inoculating guinea-pigs subcutaneously or intraperitoneally. If pure cultures are used inject 1 cc. of a broth culture. If the animals do not die, kill them at the end of 7 weeks and examine the lymphatic glands, liver and spleen for small grayish lesions resembling those of tuberculosis. Prepare smears and stain by Gram, Löffler's methylene blue and by the Ziehl-Neelsen methods. Prepare cultures as described above, incubating one set aerobically and the other with 10 per cent CO<sub>2</sub>. Isolate and identify as described above. *Br. melitensis* and *Br. suis* are more infective for guinea-pigs than *Br. abortus*.

**The Opsono-Phagocytic Test.**—Marked phagocytosis of *Brucella* in vitro by the polymorphonuclear leukocytes in whole citrated blood is regarded as evidence of immunity and an indication of recovery. Slight or no phagocytosis is indicative of susceptibility. The technic after that of Keller, Pharris, Crit and Gaub (*Jour. A.M.A.*, 1936, 107, 1369) is as follows:

1. In a test tube place 0.2 cc. of 20 per cent sodium citrate solution in physiological saline solution and add 5 cc. of blood from a vein. Mix. This gives a final dilution of 0.8 per cent citrate which prevents coagulation and inhibits normal opsonins. Use within 6 hours.

2. Shake thoroughly and place 0.1 cc. in a small test tube; add 0.1 cc. of a freshly prepared *heavy* suspension of living *Brucella* from a 24-hour agar culture in saline solution (pH 7.0). The test may be conducted with any of the three types of *Brucella* but the strain employed should be one known to be susceptible to phagocytosis.

3. Mix and incubate in a water bath at 37° C. for 30 minutes.

4. Withdraw a small amount of sedimented cells with a capillary pipet and prepare smears on slides. Dry rapidly in the air, and fix by passing through a flame.

5. Stain for 2 minutes with a 1:10 dilution of Ziehl-Neelsen's carbolfuchsin (Bondi).

6. Wash in water and dry. Examine with oil immersion objective.

7. Count the total organisms in 25 polymorphonuclear leukocytes in different parts of the field and divide by 25 to determine the average number per cell. Report as follows:

Negative:	No phagocytosis
Slightly positive:	1 to 20
Moderately positive:	21 to 40
Strongly positive:	41 or more

8. Agglutination and allergic intracutaneous skin tests with the soluble nucleoproteins are advisable at the same time:

**Examination of Cow's Milk for *Brucella Abortus*.**—1. The udder and flanks of the cow are washed with a mild antiseptic solution, dried, and the teats wiped with 70 per cent alcohol.

2. The first few streams of milk are discarded and the 20 to 25 cc. of milk collected from each quarter in sterile vials. Samples should be kept under refrigeration until examined.

3. Examine the cream by transferring 0.2 cc. of gravity cream to each of 2 Petri dishes containing 10 cc. of tryptose agar. It is advisable to add crystal violet to the media in the proportion of 1:700,000 to inhibit the growth of gram-positive organisms.

4. The cream is spread over the surface of the media by means of a sterile glass rod.

5. Incubate plates under 10 per cent CO<sub>2</sub> tension for 5 to 6 days.

6. Examine plates and transfer suspicious colonies for identification.

7. Milk samples may also be subjected to the agglutination test by adding rennin and incubating for 2 to 3 hours. Curdling will occur and then the whey will separate. The whey can be tested in the same manner as described for testing blood serum.

8. Inoculate 300 to 600 gm. male guinea-pigs. The entire cream layer from 15 cc. of milk should be injected.

## METHODS FOR THE IDENTIFICATION OF *B. TUBERCULOSIS*

1. Methods for the bacteriological diagnosis of tuberculosis of human beings include the examination of sputum, urine, gastric contents, cerebrospinal, pleural and other fluids for *B. tuberculosis* (*Mycobacterium tuberculosis*) by smears, cultures and animal inoculation.

2. The bacilli are characteristically *acid-fast* but in cultures nonacid-fast forms may be encountered.

3. Human tubercle bacilli occur as slender, straight or slightly curved rods, singly or in small clumps with the organisms at angles to each other. They may stain evenly or show granular and banded forms (Plate X, Figs. 1 and 3). They occasionally occur as threads and show branching. They are nonmotile, nonsporulating, nonencapsulated and are gram-positive.

4. Bovine tubercle bacilli (Plate X, Fig. 4) are shorter and more plump and very short forms may be intermixed with somewhat larger forms. They stain irregularly but are, likewise, acid-fast. They are less easily cultivated and are characterized by being more pathogenic for rabbits than human bacilli.

5. The tubercle bacillus grows slowly, the rate depending upon the medium employed. The colonies are ordinarily small, crumb-like, irregular, moist and later dry, yellowish-brown and with a characteristic odor. The bacilli are highly aerobic and in glycerol-broth tend to grow on the surface as a wrinkled pellicle with no turbidity and a slight granular sediment.

**Detection of Tubercle Bacilli in Sputum by Smear Examination.**—1. Pour the sputum into a Petri dish and pick up with sterilized platinum wire small white or yellow caseous particles; if none is present, choose for examination some of the thicker yellowish or greenish portions.

2. Make at least 2 smears on glass slides. They should be thin and uniform; never heavy and unevenly distributed. Material may be put on the upper half of a slide and squeezed out with another slide, continuing the rubbing until the sputum is evenly distributed when the slides are separated.

3. Stain with Ziehl-Neelsen's carbolfuchsin for acid-fast organisms.

4. The tubercle bacilli will appear as red, solid or vacuolated, straight or slightly curved rods; other bacteria and cells are stained blue. At least 2 smears should be examined before a negative report is given and 5 minutes or more devoted to the examination of each.

5. The average number of tubercle bacilli per field may be recorded according to the following scheme of Gaffky as modified by L. Brown:

No. 1, only 1 to 4 in whole preparation.

No. 2, only 1 bacillus on an average in many fields.

No. 3, only 1 bacillus on an average in each field.

No. 4, about 2 to 3 bacilli on an average in each field.

No. 5, about 4 to 6 bacilli on an average in each field.

No. 6, about 7 to 12 bacilli on an average in each field.

No. 7, about 13 to 25 bacilli on an average in each field.

No. 8, about 50 bacilli on an average in each field.

No. 9, about 100 or more bacilli on an average in each field.

No. 10, enormous numbers in each field.

6. The following are *sources of error*: (a) Scratches in the slides retaining the carbolfuchsin stain; (b) incomplete decolorization; (c) the presence of tubercle bacilli in the carbolfuchsin stain if the stain has been repeatedly used; (d) the presence of acid-fast bacilli in stale distilled water or containers; (e) wood fibers, food particles and crystals retaining the carbolfuchsin stain.

7. Examination of smears by the method of fluorescence microscopy is highly satisfactory and strongly recommended.

**Detection of Tubercle Bacilli in Urine by Smear Examination.**—1. Twenty-four-hour specimens are preferred, collected in a clean (preferably sterile) bottle.

2. Shake the specimen of urine thoroughly; fill 2 centrifuge tubes, 50 cc. capacity, and centrifuge (if smaller tubes are used more will have to be used or the centrifuging repeated until sediment from at least 100 cc. of the urine has been collected).

3. Mix the sediment from both tubes or the sediment from 100 cc. of urine. (If sediment contains crystals, they should be dissolved by very small amounts of either ammonium hydroxide if acid, or acetic acid if alkaline, diluted with water and centrifuged).

4. By means of platinum loop, transfer sediment to slides.

5. Spread out in thin films and dry.

6. Fix by gentle heat.

7. Stain for acid-fast bacilli by the Pappenheim or Ziehl-Neelsen method.

8. It may be necessary to add a drop of Mayer's egg albumin to the sediment on the slide, before spreading, to facilitate adherence.

9. An important source of error is the presence of smegma bacilli which are regarded as usually decolorized by the Pappenheim method.

# PLATE X



1. Tuberculous lymph node "giant cell" containing tubercle bacilli "human type." Bacilli are red, rest of specimen, blue. Ziehl-Neelsen stain.  $\times 1000$  diameters.

2. Tuberculous sputum from human case. Stain same as above.  $\times 1000$  diameters.

3. Tuberculous sputum; human case. Stained by Herrmann's method. Tubercle bacilli are violet, rest of specimen, brown.

4. Pus from tuberculous abscess in cow, "Bovine type" of bacillus. Stained same as 1 and 2.  $\times 1000$  diameters.

5. Section through leprous skin showing bacilli in clumps in and out of cells, and large "leprous cell" containing a ball of bacilli. Stained by Ziehl-Neelsen method.

6. Photograph of human type of tubercle bacilli from sputum. Bacilli are red, rest of specimen, blue.  $\times 1000$  diameters. (Frankel and Pfeiffer.)

(From Park, Williams and Krumwiede, *Pathogenic Microorganisms*, Lea and Febiger, Philadelphia.)





**Detection of Tubercle Bacilli in Feces by Smear Examination.**—1. Make thin smear of feces on slide or coverglass; if blood or mucus is present in the feces, this will be more apt to contain bacilli.

2. Dry in air, fix, and stain for acid-fast bacilli.

3. The concentration methods are a great aid and may be conducted in the same manner as for the examination of sputum.

4. Petroff recommends diluting the stool with 2 volumes of water, stirring and filtering to remove the coarse particles. The liquid stool is then saturated with sodium chloride crystals and allowed to stand at room temperature for several hours. Collect the scum with a sterile spoon and place in a wide-mouthed bottle. Add 2 volumes of normal sodium hydroxide, shake well and incubate at 37° C. for 1 to 2 hours. Centrifuge and decant the supernatant fluid. To the sediment add 3 to 4 drops of normal hydrochloric acid to neutralize to litmus or thymol blue. One part may be smeared on tubes of Petraghini medium, a part used for guinea-pig inoculation and a part for making smears to be stained by the usual methods.

**Detection of Tubercle Bacilli in Pleural and Spinal Fluids by Smear Examination.**—1. Collect and prepare smears as described above for urine; if a coagulum has formed, remove and tease out on a slide with pins.

2. Stain and examine for acid-fast bacilli. Prolonged search is generally required.

3. Tubercle bacilli are the only acid-fast bacilli encountered in spinal fluids. Cultures of the sediment are frequently positive when the bacilli are present. Very careful examination of smears should reveal the bacilli in 80 to 100 per cent. But in their absence the following changes, if present, should warrant a tentative diagnosis of tuberculous meningitis (Foord and Forsyth): (a) increased cell count producing a slight ground glass appearance of the fluid; (b) marked preponderance of lymphocytes but a moderate number of polymorphonuclears may show in smear-negative cases; (c) the formation of an inverted pine tree web on standing; (d) a colloidal gold curve showing maximum precipitation in the 6th or 7th tube; (e) sugar moderately or markedly reduced (averaging about 36 mgm. per 100 cc.); (f) chlorides below 650 mgm. To these criteria may be added a marked increase of protein (strongly positive Pandy reaction).

4. Petroff recommends the following method for *pleural* and *peritoneal* fluids: To approximately 10 cc. of fluid, add 2 drops of 5 per cent solution of tannic acid. Mix well and centrifuge. Decant the supernatant fluid. Prepare and stain smears of the sediment, portions of which may be also used for preparing cultures and inoculating guinea-pigs.

5. Pleural fluids containing large clots may be examined by separating the clot and adding to it equal volumes each of normal sodium hydroxide and 15 per cent solution of antiformin. Shake and digest at 37° C. for about an hour. Centrifuge and decant the supernatant fluid. To the sediment add a few drops of normal hydrochloric acid; prepare and stain smears. The clots may also be used for cultures and guinea-pig inoculation.

**Detection of Tubercle Bacilli in Milk.**—1. Use milk as fresh as possible.

2. Centrifuge 20 cc. at high speed.

3. Take off the cream on top, dilute 4 cc. with sterile water and inoculate guinea-pig subcutaneously.

4. Inject 1 to 3 cc. of sediment into additional pigs. Also prepare and stain smears.

5. Acid-fast bacilli in smears may be "butter bacilli" and also produce local lesions but not generalized infections. To prevent these errors, inoculate tubes of glycerin agar with sediment or cream. "Butter bacilli" develop in a few days at 37° C. and also at room temperature.

6. When ready to examine the pigs, inoculate each with 2 cc. of old tuberculin late in the day. The following morning the tuberculous animals will be dead or dying; conduct autopsies to confirm the results.

**Detection of Tubercle Bacilli by Concentration Methods.—Antiformin Method.**—This is considered useful when direct smears are negative. Examination of a sample of the entire 24-hour sputum is recommended. Antiformin is a proprietary preparation and may be prepared by mixing equal parts of liquor sodae chlorinatae, U. S. P. and a 15 per cent solution of sodium hydroxide. The former is prepared by dissolving 600 grams of sodium carbonate and 400 grams of chlorinated lime in 4000 cc. of distilled water, allowing the mixture to settle and then filtering the supernatant fluid. Equal parts are mixed with a 15 per cent solution of sodium hydroxide. Keep in a dark bottle in a cool place.

1. Place equal parts of sputum and 50 per cent antiformin solution in small beaker or prepare the mixture in the sputum container.

2. Incubate at 37° C. for 30 minutes, stirring occasionally to insure complete liquefaction or shake for 30 minutes in a shaking machine.

3. Dilute with 3 volumes of sterile water to reduce specific gravity of the solution.

4. Centrifuge for 10 to 30 minutes; pour off supernatant fluid; fill tube with water; centrifuge; continue until all of the fluid has been centrifuged.

5. To sediment left, after pouring off supernatant fluid, add sterile distilled water, mix well, centrifuge and pour off supernatant fluid.

6. By means of a platinum loop transfer sediment to slides.

7. Make smears and stain for acid-fast bacilli.

8. If the sediment does not adhere to the slide, apply a thin smear of Mayer's albumin or raw egg albumin to the slide (egg white, 1 part; water, 10 parts; formalin, 1 part) and spread the sediment.

9. The sediment may be used for cultural purposes or for inoculation of guinea-pigs, although many tubercle bacilli may be destroyed.

**Petroff's Method.**—Instead of using antiformin, Petroff recommends adding to the sputum an equal volume of 4 per cent sodium hydroxide. The mixture is kept at 37° C. for 15 to 30 minutes, shaking frequently to insure a uniform mixture. After complete homogenization, it is centrifuged at high speed and the supernatant fluid decanted. To the sediment add 2 or 3 drops of normal hydrochloric acid to make it slightly acid and prepare smears as above.

With *urine* Petroff's method is conducted with 24-hour specimens as follows: acidulate with a few drops of 30 per cent nitric acid. To every 1000 cc. add 2 cc. of 5 per cent tannic acid solution. Shake well and place in refrigerator for 24 hours. Decant the supernatant fluid and centrifuge the sediment; decant the supernatant fluid. Treat the sediment with 1 cc. of normal sodium hydroxide solution, which should completely dissolve it. Warm for 30 minutes at 37° C.; dilute with 3 volumes of sterile water and centrifuge. Decant, prepare and stain smears of the sediment. If cultures are to be made, add 5 volumes of normal sodium hydroxide to the sediment; shake well, incubate for 30 minutes and centrifuge at high speed for 5 to 10 minutes (some

bacilli are destroyed). Decant the supernatant fluid, add 2 drops of normal hydrochloric acid, mix well and distribute the sediment on the surface of tubes of medium containing one of the static dyes. Paraffin the stoppers and incubate. Tubercle bacilli if present will appear in about 2 to 10 weeks.

*Gerundo's method* (*Jour. Lab. and Clin. Med.*, 28: 328, 1943) employs a fluid prepared of 1 gm. pepsin, 10 cc. glycerin, 15 cc. hydrochloric acid and 1 gm. sodium fluoride in 1000 cc. distilled water. Equal volumes of sputum or other material and the fluid are mixed and incubated at 37° C. for 4 hours with occasional shaking. Centrifuge, wash with saline solution several times, prepare smears of the sediment and inoculate tubes of Petraghini's or other medium.

**Methods for the Cultivation of Tubercle Bacilli.**—Inoculate slants of Petraghini's medium with sediment obtained by concentration methods described above. Petroff's and Dorset's egg medium may be employed, but the Petraghini medium is preferred. If the material is concentrated by using a strong acid instead of alkali, the sediment should be rendered neutral to litmus with decinormal hydrochloric acid before inoculating culture media. The culture tubes should be capped with tinfoil or a sterile cork after the cotton plugs have been impregnated with hot paraffin to prevent drying of the medium.

The *method of Corper and Uyei*, employing their potato medium (page 359) is as follows: 1. One cc. of sediment is beaten to a homogeneous pulp and introduced into a sterile centrifuge tube of 15 cc. capacity with 1 cc. of 6 per cent sulphuric acid (17 cc. of 96 per cent (specific gravity, 1.84) sulphuric acid in 500 cc. of distilled water) or, better, 1 cc. of 5 per cent pure oxalic acid (by weight). After incubation at 37° C. for 30 minutes, neutralize with normal sodium hydroxide, checking the reaction with litmus. Add about 10 cc. of sterile 0.9 per cent sodium chloride solution and centrifugalize. The residue, after the supernatant fluid has been decanted, is seeded lightly on the surface of 3 to 6 tubes of the medium, the culture tube being capped with tinfoil or a sterile cork after the cotton plug has been lightly impregnated with hot paraffin to prevent drying out of the medium.

2. The culture tubes should be incubated in the dark, with due precaution being taken to avoid drying of the medium or contamination. A luxuriant growth should occur on this medium within from 2 to 6 weeks; but if the culture is negative, the tubes should not be discarded for diagnostic purposes until after 3 months' observation at incubator temperature.

3. A few of the ordinary precautions necessary in growing tubercle bacilli by this method are: (a) Avoid changes which may occur in the potatoes before autoclaving by not allowing them to stand too long a time after cutting. (b) Avoid drying of the culture medium during the long period of incubation necessary by paraffining the cotton stoppers. (c) Keep the culture tubes, while in the incubator, in a dark receptacle like a covered tin can or keep the incubator dark, preferably both. (d) Maintain a constant temperature of 37.5° C. (e) pH of medium 7.0 with isotonicity. (f) Eliminate secondary organisms. (g) Avoid inhibitory dyes in the medium.

Tubercle bacilli in exudates or other material contaminated with other organisms may be isolated by injecting the material into a guinea-pig and then obtaining material aseptically from the tuberculous lesions. Such material will contain many more tubercle bacilli and none of the contaminating organisms. Otherwise the tubercle bacilli grow slowly and may be readily overgrown by contaminating organisms.

**Detection of Tubercle Bacilli by Animal Inoculation.**—1. Morning *sputum* is preferred and inoculated at once or after washing with sterile saline solution. If not fresh and if many other bacteria are present, it may be treated with an equal volume of 6 per cent sulphuric acid and neutralized with about an equal volume of 5 per cent sodium hydroxide to avoid septic infection of the animals. If *urine* is to be examined, collect sediment from 100 cc. *Feces* should be digested in the same manner as sputum.

2. Weigh 2 guinea-pigs and note their weights. Also note their color or otherwise mark them for further identification.

3. Inoculate each pig subcutaneously with the sediment from 50 cc. of urine, a bean-sized portion of sputum (about 0.5 gm.) or the washed sediment of feces after treatment with sodium hydroxide and oxalic acid as described above. Do not inject into the mammary glands.

4. Bloch recommends damaging the inguinal lymph glands by squeezing between the finger, and injection of the material into these damaged glands (of questionable value).

5. Examine the animals each week for symptoms of tuberculosis. Weigh them and examine site of inoculation for tubercle or tuberculous ulceration; also superficial lymph glands or enlargements. If the subcutaneous glands are enlarged, obtain pus and examine by smears for acid-fast bacilli since saprophytes are destroyed in a few weeks while tubercle bacilli multiply.

6. If the animals show physical signs of tuberculosis at the end of 2 weeks, one may be killed and the presence of tuberculosis confirmed, in which case the other animal may be destroyed and examined. If necropsy of first animal fails to reveal tuberculosis, allow the other to live for 4 to 6 weeks, then destroy, examine and report positive or negative findings. Or at the end of 2 weeks inject one of the animals intracutaneously with 0.1 cc. of a 5 per cent solution of old tuberculin. If no reaction has supervened within 48 hours the test should be repeated 2 weeks later.

7. Should neither pig show any signs of tuberculosis, one should be allowed to live 4 to 6 weeks before destroying and the other for a longer period up to at least 3 months. It sometimes happens that specimens of urine contain organisms other than tubercle bacilli in large numbers which cause the death of the inoculated animals in a few days and require a repetition of the test.

8. Enlarged glands or tissue from other organs (spleen) may be examined for tubercle bacilli by making smears and staining. If acid-fast bacilli are not found, the tissues should be examined histologically for tuberculosis before a negative report is warranted. Smears made from the site of inoculation are especially important.

9. Never base a negative report alone on the absence of enlarged external glands; the internal glands and spleen require examination.

10. Guinea-pigs kept in cages in a room free from tuberculous animals and cared for by healthy caretakers rarely develop spontaneous tuberculosis.

11. While cultural methods are equally valuable, the guinea-pig inoculation test when properly conducted is an excellent method for proving the presence of virulent tubercle bacilli in material that is either known or suspected of containing acid-fast bacilli.

**METHODS FOR THE IDENTIFICATION OF B. SMEGMATIS**

1. This organism is a nonpathogenic saprophyte which may occur in the smegma about the genitalia of both sexes and since it is also acid-fast, may be encountered in urine and thus mistaken for *B. tuberculosis*.

2. It is similar in morphology to the tubercle bacillus but more pleomorphic, sometimes occurring in short comma forms and occasionally as spirals.

3. It is at times cultivated with great difficulty, the media requiring enrichment with serum or hydrocele fluid.

4. For differentiation from the tubercle bacillus, smears should be stained by the Pappenheim method. According to Cole, smegma bacilli may resist decolorization for 4 hours at most, while tubercle bacilli will retain the stain for as long as 24 hours, although this differentiation is not absolutely dependable.

5. The final and more conclusive test for differentiation is by guinea-pig inoculation since *B. smegmatis* is nonpathogenic; or by the cultural method with 6 per cent sulphuric or 5 per cent oxalic acid which destroy saprophytic acid-fast bacilli, but not tubercle bacilli.

6. While in the great majority of instances acid-fast bacilli in urine are tubercle bacilli, yet smears stained by the Pappenheim method should be used and all specimens showing acid-fast bacilli subjected to the inoculation test.

**METHODS FOR THE IDENTIFICATION OF B. LEPRAE**

1. Leprosy is caused by *B. leprae* (*Mycobacterium leprae*) and is detected by the examination of smears or sections of nodules stained by acid-fast technic. Neither cultures nor animal inoculation are of any aid in laboratory diagnosis.

2. Since the initial lesion is often an ulcer of the mucosa of the nose, prepare smears and stain by the method of Ziehl-Neelsen. If desirable, the patient may be given 60 grains of potassium iodide beforehand to produce coryza and increase the nasal secretions. Saprophytic acid-fast bacilli may be mistaken for *B. leprae*.

3. Prepare smears of a skin lesion with a safety razor blade or scalpel and stain by method of Ziehl-Neelsen.

4. If possible remove a portion of lesion by biopsy and place in 4 per cent formalin. Prepare paraffin sections and stain for acid-fast bacilli.

5. *B. leprae* are acid-fast (Plate X) and gram-positive. They are rather long, slender and usually straight with pointed ends. Decolorization should not be carried too far as they are more easily decolorized than *B. tuberculosis*.

6. In nasal smears the bacilli are apt to be packed in cells (lepra cells) while in sections of nodules they are found chiefly in the skin, packed in characteristic lepra cells (foam cells) and in the endothelium lining the lymphatics.

7. Lepra bacilli are rarely demonstrable in the anaesthetic type of nerve leprosy.

**METHODS FOR THE IDENTIFICATION OF BACTERIUM COLI**

1. *Bacterium coli* (*Escherichia coli*) is a normal inhabitant of the intestinal tract of man and all vertebrates. At least 15 species have been identified in feces and soil, some of which have been found in milk and cheese. Only 2 are of special interest in human beings, namely, *B. coli communis* and *B. coli communior*.

2. So many occur in the feces that there is no difficulty in isolation although identification requires special methods for differentiation from other gram-negative, motile bacilli of similar morphology.

3. The material usually submitted for examination for *B. coli* comprise the following: (a) *Urine* from cases of cystitis and pyelitis; (b) *bile* from cases of suspected biliary tract disease; (c) *peritoneal* exudates in peritonitis; (d) *pus* from wounds, abscesses, the prostate gland and fistulae, especially in the region of the rectum, urethra and kidneys; (e) *blood cultures* in suspected septicemia and, (f) occasionally *cerebrospinal fluid* in suspected meningitis. The bacteriological examination of water

includes tests for *B. coli* from the standpoint of possible fecal contamination; special methods are employed as described on page 509.

4. The bacterium grows readily in ordinary media so that cultures of urine, bile, peritoneal exudates and pus of other sources, blood cultures, etc., on plain or blood agar produce circular, raised, low convex, smooth, white to yellowish, finely granular, moist, buttery colonies which are not adherent, easily emulsified and with entire or undulating edges. It is better, however, to prepare surface streak plates on the Endo medium on which the colonies of *B. coli* are pink to red with a metallic sheen. Or the material may be streaked on plates of eosin methylene-blue agar on which *B. coli* produces large discrete colonies with large, dark, almost black centers, with



FIG. 194.—*BACILLUS COLI COMMUNIS*

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

a greenish metallic sheen. The use of either of these special media immediately facilitates the identification of the organism. On desoxycholate agar the colonies are large and uniformly red. On desoxycholate citrate agar *B. coli* usually fails to grow unless the inoculum is very heavy, in which case the colonies are identical with those on desoxycholate agar. *B. coli* is motile.

5. Direct smears of such materials and of colonies stained by the Gram method show short, gram-negative rods (Fig. 194) with no spores and no capsules. Their morphology, however, is not characteristic so that it is impossible to identify and differentiate *B. coli* from other bacilli of the typhoid-colon group by morphology alone.

6. Transplant suspicious colonies to the slopes and butts of tubes of Russell's double sugar agar. After incubation at 37° C. for 24 to 48 hours, *B. coli* produces acid on the slopes with acid and gas in the butts.

7. For final identification inoculate litmus milk in which *B. coli* produces acid; peptone water in which indol is produced and fermentation tubes of dextrose and saccharose broths. *B. coli communis* produces acid and gas in dextrose while *B. coli* *communior* produces acid and gas in both dextrose and saccharose (see Table 21).

Species	Motility	Lactose	Mannite	Dextrose	Maltose	Xylose	Rhamnose	Sucrose	Dulcitol	Gelatin	Milk	Indol	Voges-Proskauer	Hydrogen sulfide	Russell's Medium	
															Butt	Slant
<i>B. coli communis</i>	+	G	G	G	G	+	G	—	+	—	ac	+	—	—	G	a
<i>B. coli communior</i>	+	G	G	G	G	G	G	G	+	—	ac	+	—	—	G	a
<i>B. acidilactici</i>	—	a	a	a	a	x	x	x	+	x	x	+	+	x	x	x
<i>B. lactis aerogenes</i>	+	G	G	G	G	G	G	G	+	—	ac	—	—	—	G	a
<i>B. friedländeri</i>	+	+	x	G	G	G	G	G	+	—	a	±	x	—	G	a
<i>B. cloacae</i>	+	G	G	G	G	G	G	G	+	+	ac	±	+	—	G	ak
<i>B. typhosus</i>	+	—	a	a	a	—	—	—	—	—	ak	—	—	+	a	k
<i>B. faecalis alcaligenes</i>	+	—	—	—	—	—	—	—	—	—	k	—	x	+	k	k
<i>B. proteus-vulgaris</i>	+	—	—	a	a	a	x	x	—	x	x	±	—	x	x	x
<i>S. paratyph. A</i>	+	—	G	G	G	—	G	—	G	—	a	—	—	—	G	k
<i>S. paratyph. B</i>	+	—	G	G	G	G	G	—	G	—	ak	—	—	+	G	k
<i>S. paratyph. C</i>	+	—	x	G	G	G	G	—	G	—	ak	—	—	+	G	k
<i>S. enteritidis</i>	+	—	G	G	G	G	G	—	G	—	ak	—	—	+	G	ak
<i>S. morganii</i>	+	—	—	G	G	G	a	—	±	—	k	+	x	+	G	k
<i>S. cholera-suis</i>	x	—	—	G	G	G	G	—	G	—	ak	—	—	+	G	ak
<i>S. abortivo-equinus</i>	+	—	—	G	G	G	G	—	G	—	ak	—	—	+	G	ak
<i>S. aertrycke</i>	+	—	G	G	G	G	G	—	G	—	ak	—	—	+	G	ak
<i>S. pullorum</i>	—	—	G	G	G	G	G	—	G	—	ak	—	—	+	G	ak
<i>S. gallinarum</i>	—	—	G	G	G	G	G	—	G	x	ak	—	—	+	G	x
<i>S. suipestifer</i>	+	—	a	+	a	a	a	—	a	x	ak	—	—	—	x	x
<i>B. dysenteriae (Shiga)</i>	—	—	a	a	a	a	x	x	a	x	x	—	—	—	x	x
<i>B. dysenteriae (Flexner)</i>	—	—	a	a	a	—	a	—	a	—	ak	—	—	—	a	k
<i>B. paradyserteriae ("y")</i>	—	—	a	a	a	a	a	a	a	x	ak	—	—	—	a	k
<i>B. paradyserteriae (Strong)</i>	—	x	a	a	—	—	—	x	—	x	x	+	—	—	x	x
<i>B. sonnei</i>	—	a	x	a	a	—	a	a	—	—	ac	—	x	—	x	x

G = acid and gas; a = acid only; ac = acid and coagulation; a = acid or negative.

+ = positive (indol production, liquefaction of gelatin or formation of hydrogen sulfide).

— = no change; ± = acid and gas or negative; k + alkaline; ak = acid to alkaline; x = not needed for identification.



**METHODS FOR THE IDENTIFICATION OF *B. PROTEUS-VULGARIS***

1. *B. proteus-vulgaris* is commonly found in putrefying animal and vegetable materials as well as in the soil and feces. Its primary pathogenicity is slight, but it may be a source of important secondary infection in gunshot and other wounds, cystitis, pyelitis and of other mucous membranes as well as possibly producing a type of food poisoning.

2. Materials submitted for its examination are usually feces, urine and pus which may be plated in the same manner as described for the typhoid bacillus.

3. The organism occurs as a straight or slightly curved rod with rounded ends varying greatly in size and shape, singly, in pairs or in chains.

4. Most strains are actively motile; all are noncapsulated, nonsporulating and gram-negative.

5. It grows rapidly on plain agar at 37° C. with the production of thin, bluish-gray colonies that spread rapidly over the entire plate designated by Weil and Felix as H ("Hauch" = film) colonies. Nonmotile variants grow in denser, round colonies designated as O ("Ohne Hauch" = without film).

6. On plain blood agar the colonies appear as a thin, shiny, gray film covering the entire surface of the medium. Usually this spreading growth causes complete hemolysis of the blood. Two types of swarming occur. In the more common type the bacilli spread in a film which shows a more or less rippled appearance. Less frequently encountered are strains which spread in definite concentric rings, the edges of which are well defined and may be quite regular. Often plates which are overgrown with *Proteus* have a characteristic odor.

7. The growth of a *Proteus* on desoxycholate agar plates may be mistaken for intestinal pathogens. Since this medium almost entirely inhibits spreading, colonies of *Proteus* are small, colorless, and resemble those of the *Salmonella*, *Eberthella* and *Shigella* groups. Occasionally strains are encountered in which some slight spreading occurs, in which case they appear as definite colonies surrounded by an irregular zone of sparse secondary growth.

8. *Proteus* often occurs in mixed culture. When other gram-negative bacilli are present, desoxycholate agar may be used for their isolation. If the concurring organisms are gram-positive cocci, however, the most satisfactory medium is blood or ascitic agar containing chloral hydrate as follows: (a) Add 0.25 cc. of 5 per cent solution of chloral hydrate to a tube of melted agar plus blood; (b) pour into a sterile Petri dish and when hard streak the surface with the culture. On this medium there is usually no inhibition of the growth of gram-negative bacilli but the spread of *Proteus* is prevented with the development of well-isolated, discrete colonies.

9. Pick off suspicious colonies and transplant to slants of plain or blood agar for further identification.

10. The organism digests Löffler's blood serum and gelatin. All strains produce acid and gas in dextrose, galactose and sucrose (may be lost by old strains) and most in salicin and maltose. None ferment lactose, mannitol or mannose. Litmus milk is first rendered slightly acid and then markedly alkaline. Indol is produced by some strains. The Voges-Proskauer test is negative (see Table 21).

11. Cultures of the X19 strain are used in the Weil-Felix agglutination test for typhus fever. It should be of the nonmotile or O dissociative type.

**METHODS FOR THE IDENTIFICATION OF *B. TYPHOSUS***

1. The materials usually examined for *B. typhosus* (*Eberthella typhosus*) are (1) blood cultures; (2) urine; (3) feces; (4) bile and (5) occasionally pus, cerebrospinal fluid, etc.

2. The organism occurs as rods of varying length (Fig. 195), usually singly, but sometimes in pairs and short chains; nonsporulating; nonencapsulated and highly motile. In broth cultures incubated at room temperature the bacilli are usually long and slender, but it is impossible to identify *B. typhosus* by morphology alone. It stains readily and is gram-negative.

3. The colonies of freshly isolated *B. typhosus* on plain or blood agar are usually round with moderate domes, grayish, transparent to opaque, with entire edges. On special media the colonies vary according to the medium employed.

**Blood Cultures.**—1. These may be prepared in nutrient broth, Kracke medium or bile broth. A simple broth containing 0.05 to 0.3 per cent sodium desoxycholate with a pH of 6.0 to 8.0 is stated by Grubb to inhibit staphylococci without a significant inhibition of typhoid bacilli.

2. Incubate at 37° C. and examine daily. Prepare smears stained by the method of Gram.

3. If gram-negative motile bacilli are found inoculate plates of eosin-methylene blue agar or desoxycholate citrate agar by the surface streak method. Incubate at 37° C.

4. On eosin-methylene blue agar plates colonies of *B. typhosus* are translucent, colorless or pinkish. On desoxycholate citrate agar the colonies are colorless or almost colorless.

5. Prepare smears and stain by the Gram method. If gram-negative bacilli are present inoculate the butt and slant of Russell's double sugar agar and incubate for identification as described later.

6. If there is no growth within 10 days the culture may be reported sterile. Positive cultures usually show the organisms within 3 days.

**Urine Cultures.**—1. It is always advisable to collect urine aseptically by catheterization and if *B. typhosus* is present it is usually to be found in pure culture. Urine collected in the ordinary way is almost sure to show *B. coli*, staphylococci and other organisms.

2. With a sterile pipet transfer 2 to 5 cc. to a flask of nutrient broth. Also inoculate the surface of an eosin-methylene blue plate with 1 or 2 cc.

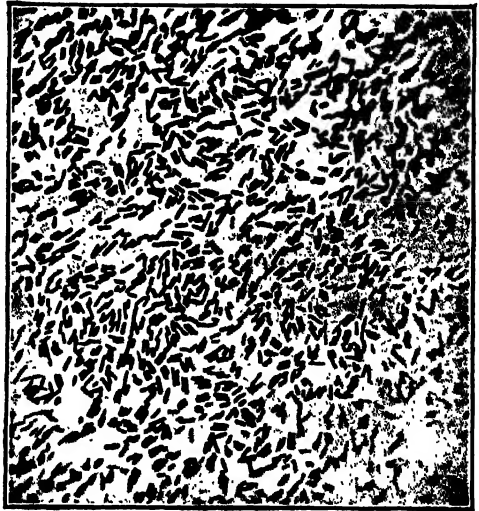


FIG. 195.—*BACILLUS TYPHOSUS*

From twenty-four-hour culture on agar, showing regularity of forms. (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

3. Incubate for 48 to 72 hours. If there is no growth in this period the specimen may be reported as sterile.

4. If a growth appears examine smears stained by the Gram method. On the eosin methylene-blue medium colonies of *B. typhosus* are translucent, colorless or pinkish. Subculture on slants of Russell's double sugar agar and conduct further tests for identification as described later.

5. Another excellent method which is useful if the urine is likely to be contaminated with *B. coli* and other organisms is as follows:

Inoculate 10 cc. of selenite-F enrichment medium with 5 cc. of urine and incubate for 24 hours. The selenite inhibits the growth of *B. coli* during this interval but allows typhoid bacilli to proliferate. Prepare surface streak plates of desoxycholate citrate agar and incubate for 24 hours. *B. coli* produces deep pink colonies with a halo due to the precipitation of sodium desoxycholate. Colonies of *B. typhosus* are fairly large, translucent, colorless or bluish with slightly granular structure; they are usually readily detected.

**Bile Cultures.**—1. Bile may be aspirated from the gallbladder or collected by the method of Lyon as described on pages 210 to 215.

2. With a sterile pipet transfer 1 to 3 cc. to a flask of nutrient broth and incubate at 37° C.

3. Examine daily and if there is no growth over a period of 5 to 7 days the specimen may be reported as sterile.

4. If a growth appears examine smears stained by the method of Gram. Also inoculate plates of eosin-methylene blue agar and tubes of Russell's double sugar agar. For the latter use a long platinum needle, which is first plunged into the butt of the medium and then lightly drawn over the surface of the slant. Conduct further tests for identification as described later.

**Methods for the Isolation of *B. Typhosus* from Feces.**—1. Various special media are employed to inhibit the growth of fecal organisms and especially *B. coli* while permitting *B. typhosus* to survive and proliferate in more or less characteristic colonies. The feces should be fresh, as standing for as long as 12 hours may diminish positive findings by 50 per cent.

2. Thoroughly emulsify a small amount of feces in a tube (10 cc.) of selenite-F enrichment medium and incubate 18 to 24 hours. Streak 1 loopful on a plate of *desoxycholate citrate agar* and 2 loopsful on a second. Incubate 24 hours. Colonies of *B. typhosus* are large, translucent, granular and colorless or bluish. Colonies of *B. coli* are of a deep pink color surrounded by halos due to precipitation of sodium desoxycholate.

3. Another good medium is *eosin-methylene blue agar* in large plates allowed to harden and dry by standing for a few hours before use.

Prepare a suspension of about 1 part of feces in 25 parts of sterile saline solution and allow to stand until the large particles have settled.

With a sterile bent glass rod dipped into the emulsion, inoculate a plate by beginning at the center and passing outward in concentric circles until the entire surface has been gently smeared. A second plate should be inoculated in the same way without redipping.

Incubate at 37° C. for 24 hours. Colonies of *B. typhosus* are usually round with high domes, translucent, colorless or pinkish with entire edges.

Pick off suspicious colonies and inoculate the butts and slants of Russell's double sugar agar for further identification as follows:

**Methods for Identifying *B. Typhosus*.**—1. Incubate the subcultures on Russell's double sugar agar at 37° C. for 24 to 48 hours. *B. typhosus* produces acid but no gas in the butts with the slant unchanged (alkaline).

2. With such results prepare smears and stain by method of Gram. Gram-negative bacilli are presumably typhoid bacilli but further tests are required.

3. Inoculate fermentation tubes of dextrose, maltose, mannite and other broths (see Table 21).

4. Incubate 24 to 48 hours. *B. typhosus* produces acid but no gas with dextrose, maltose and mannite but not with lactose.

5. Finally *agglutination tests* may be conducted by a *rapid slide* method as follows:

(a) For each colony or slant to be tested, arrange coverglasses and place on each a small drop of suitable dilution of rabbit-immune serum capable of giving strong agglutination with *B. typhosus*. As a general rule this may be approximately 1:100.

(b) Emulsify in a drop of diluted serum a small portion of the colony to be studied picked up with a platinum loop. Number the colonies to correspond to the slides.

(c) At the same time prepare controls of each colony in the same manner, using saline solution instead of serum.

(d) Suspend each preparation in hanging drop slides.

(e) Examine microscopically in about 15 minutes. Strong agglutination is presumptive evidence of a colony being typhoid bacilli.

(f) Or place drop of diluted serum on a plain slide and emulsify in each a very small amount of the colonies to be tested. Tilt the slide back and forth for a few seconds over a black background. Agglutination is readily detected by the development of a granular appearance.

If a *test tube method* is preferred it may be conducted as follows:

(a) Arrange 6 small test tubes and place 1 cc. of distilled water in each.

(b) To No. 1 add 1 cc. of a 1:25 dilution of typhoid-immune serum and mix well.

(c) Transfer 1 cc. to No. 2; mix well and transfer 1 cc. from No. 2 to No. 3 and so on to No. 5, from which 1 cc. is discarded. The dilutions are 1:50, 1:100, 1:200, 1:400 and 1:800. No. 6 is the control.

(d) To each tube add 1 cc. of a heavy broth culture of the organism which gives final dilutions of 1:100 to 1:1600.

(e) If the organism is on a solid medium it may be washed off and emulsified in saline solution to give a suspension of approximately 2,000,000,000 per cc. and used instead of a broth culture. Or the growth may be removed from an agar slant with a platinum loop and emulsified direct in the serum dilution; this, however, gives final dilutions of 1:50 to 1:800.

(f) Mix well and place in a water bath at 50 to 55° C. for 2 hours and then in a refrigerator overnight when the readings are made.

When agglutination tests are indefinite or if cross-agglutination occurs, it is possible to identify the organism by *absorption tests* conducted as follows:

1. Inoculate pint Blake bottles of agar with each culture to be used.

2. After 18 to 24 hours suspend each growth in from 5 to 10 cc. of saline solution.

3. Centrifugalize each at high speed for 30 to 60 minutes.

4. Remove the supernatant fluids and add 5 to 10 cc. of 1:50 typhoid or other immune serum to each sediment.
5. Mix well and place in a water bath at 45° C. for 3 hours, shaking occasionally, and then in the refrigerator overnight.
6. Centrifugalize at high speed for 30 to 60 minutes.
7. Prepare dilutions of 1:100, 1:200, 1:400, 1:800, and 1:1600 of each supernatant fluid and place 0.5 cc. of each in small test tubes. In tube No. 6 place 0.5 cc. of saline solution for control.
8. Add 0.5 cc. of a heavy broth culture or saline suspension of one of the cultures of each tube.
9. Set up duplicate tests with the second or remaining cultures to be tested.
10. Mix well and place in a water bath at 37° C. for 2 hours and in the refrigerator overnight when the readings are made.
11. If a typhoid serum is employed, the cultures completely absorbing the agglutinin (negative reactions) are of typhoid bacilli.

### METHODS FOR THE IDENTIFICATION OF PARATYPHOID BACILLI

1. Paratyphoid bacilli belong to the genus *Salmonella*. Infections with them constitute the Salmonellosis. More than a hundred bacilli of the genus have been identified.
2. *Salmonella* occur not only in man, but in the lower animals as well (including birds). Human beings may be infected with the *Salmonella* of the lower animals through the contamination of foods and water, but it is likely that most infections are due to bacilli of human origin.
3. *Salmonella* infections may occur sporadically, in epidemics, or in carriers without symptoms. Most epidemics are food-borne. Persistent convalescent carriers and unrecognized cases are the most important sources of infection.
4. Paratyphoid fever is usually caused by *Salmonella paratyphi B* (*Salmonella schottmülleri* or *B. paratyphosus B*). A smaller percentage is due to infection with *Salmonella paratyphi A* (*B. paratyphosus A*).
5. About 60 per cent of *Salmonella* infections, however, are characterized by gastro-enteritis due to food poisoning. These food infections are due mostly to *S. aertrycke* (*S. typhi-murium*), *S. enteritidis* (*B. enteritidis*), *S. newport* and *S. oranienburg*. *S. cholera-suis* is only rarely responsible. *S. morgani* is of doubtful pathogenicity, but has been found in the diarrheal stools of infants and in the feces of adults with "asylum dysentery". *S. suispestifer* may not only produce food poisoning but other infections like septicemia, osteomyelitis, endocarditis and pericarditis. *B. faecalis alcaligenes* and *B. lactis aerogenes* are not pathogenic, but of interest and importance from the standpoint of being mistaken for *Salmonella*.
6. When investigating an outbreak of food poisoning (a) secure leftover portions of the suspected food, pack in ice and examine as soon as possible; (b) secure specimens of vomitus and feces for bacteriological examinations, and at a later date, samples of patient's serum for agglutination tests; (c) try to determine the source of the food and secure feces for bacteriological examination and blood for agglutination tests from the handlers and especially cooks suspected as carriers and (d) specimens of blood, spleen, liver and intestines from fatal cases for bacteriological examination.

7. Prepare smears of the foods and stain by Gram to obtain some idea of the predominating organisms.

8. All *Salmonella* occur as gram-negative bacilli which cannot be differentiated from other members of the colon-typhoid-dysentery bacilli by morphological characteristics.

9. The methods of examination are exactly the same as described for the isolation and identification of the typhoid bacillus. Surface streak cultures on plates of eosin-methylene-blue agar and desoxycholate citrate agar are recommended for purposes of isolation.

10. Suspicious colonies should be fished and pure cultures subjected to study in carbohydrate and other media, as indicated in Table 21.

11. Final identification requires the aid of agglutination tests. Species specific immune sera are required. These are prepared by the immunization of rabbits with formalinized (0.3 per cent) broth cultures of motile bacilli from smooth colonies, three intravenous injections of 0.5, 1.0 and 2.0 cc. at intervals of 5 to 7 days being usually sufficient. The sera may be preserved with 0.5 per cent phenol. Type specific antigens are prepared by removing group agglutinins by adsorption.

12. In conducting agglutination tests two antigens of the culture are employed. The somatic antigen may be prepared by emulsifying the growth from an 18- to 24-hour agar slant culture in 1 cc. of absolute ethyl alcohol. Heat to 60° C. for 1 hour to inactivate the flagellar components, centrifuge thoroughly and discard the supernatant fluid. Suspend the sediment in 0.5 cc. of saline solution. Make the test by mixing a drop of antigen with a drop of the typing serum on a glass slide and tilting it back and forth for 1 or 2 minutes. Read agglutination macroscopically.

Flagellar antigen is prepared by adding to an 18- to 24-hour actively motile broth culture an equal volume of saline solution containing 0.6 per cent formalin. For the test, 1 cc. of the antigen is added to a small test tube containing 0.01 cc. of the type serum and the mixture incubated at 50 to 55° C. The tube is examined for floccular agglutination at the end of 1 hour and again after 2 hours. If tests are incubated for more than 2 hours, somatic agglutination may yield confusing results.

## METHODS FOR THE IDENTIFICATION OF DYSENTERY BACILLI

1. Bacillary dysentery is caused by Shiga's *B. dysenteriae* (*Shigella dysenteriae*) or by the paradysentery bacilli listed in Table 21.

2. As the organisms do not produce septicemia, blood cultures are not employed. The material submitted for examination should be *fresh* fecal discharges and preferably the blood-stained mucopurulent portions.

3. Callendar states that a presumptive diagnosis can be usually made by the microscopic examination of stained and unstained coverglass preparations of very fresh material for a study of the cells. Blood is present in varying amounts. Neutrophils form about 90 per cent of the exudate, many showing nuclear degeneration (ringing) with fat in the cytoplasm. Endothelial macrophages are found containing erythrocytes and leukocytes. They undergo toxic degeneration and form "ghost cells." Plasma cells are abundant early in the disease.

4. The dysentery bacilli occur as rods or coccobacilli and usually singly. They are nonmotile, noncapsulated and nonsporulating. They stain readily and are gram-

negative. Since their morphology and staining are not characteristic, the examination of smears of material possess no diagnostic value.

5. A good preserving fluid for swabs secured by proctoscopic examination may be prepared according to Felsenfeld (*Jour. Lab. and Clin. Med.*, 28: 1255, 1943) by dissolving 10 gm. sodium citrate, 10 gm. peptone, 5 gm. sodium desoxycholate and 9 gm. sodium chloride in 1000 cc. distilled water. Add 3 cc. of a 1 per cent monosodium phosphate solution and neutralize with decinormal solution of sodium hydroxide. Add 0.05 gm. p-aminobenzoic acid. Adjust to pH 8.0, tube and sterilize in the Arnold.

6. Prepare surface streak plates of eosin-methylene blue agar using flecks of blood-stained mucopurulent material. Incubate at 37° C. for 24 hours. Colonies of *B. dysenteriae* (Shiga) are small, round, translucent, pinkish or colorless. Endo medium may be used, but since dysentery bacilli do not ferment lactose the colonies are colorless and this medium sometimes inhibits their growth. Blood agar plates may be employed. Desoxycholate-citrate agar plates are very satisfactory.

7. Transfer suspicious colonies of gram-negative bacilli to agar slants and broth. Incubate 24 hours and examine for motility and by gram stain.

8. For identification inoculate peptone water for indol production, litmus milk and fermentation tubes of dextrose, mannite, lactose and dulcitol for acid (gas not produced). The differential characteristics of the important members of the group are shown in Table 21.

9. *B. dysenteriae* (Shiga) and the other members of the group produce slight acidity in litmus milk followed by neutrality or slight alkalinity except in the case of *B. alcalescens*, where the medium becomes very alkaline.

10. Final identification usually requires agglutination tests with known anti-dysentery serum. The technic is the same as described under the typhoid bacillus. Polyvalent serum may be employed or monovalent sera for the respective types. The dilutions to employ depend upon the titers of the sera.

## METHODS FOR THE IDENTIFICATION OF *VIBRIO CHOLERAE*

1. The cholera vibrio (*Vibrio cholerae*) occurs in the stools of patients with Asiatic cholera and carriers; also sometimes in the vomitus of this disease as well as in contaminated water, milk or other foods.

2. The "rice water" stools are preferred; do not add glycerin as a preservative. The feces of suspected carriers may be used or swabbings may be taken from the rectum.

3. The organism occurs as slightly curved rods with rounded ends, often resembling a comma (Fig. 196). They occur singly, in S pairs, short chains or spirals. In old cultures they may be small, granular and stain poorly. Involution forms are frequent.

4. They are very actively motile, noncapsulated and nonsporulating.

5. They stain best with carbolfuchsin and are gram-negative.

6. On plain agar, blood agar or Dieudonné's alkaline blood agar, the colonies are round, low convex, translucent, finely granular ("heaped glass"), buttery and grayish-yellow with entire edges and surrounded by a zone of alpha or beta hemolysis.

7. It rapidly produces indol and reduces nitrates (cholera red reaction), liquefies gelatin, grows abundantly in broth with a thick pellicle being strongly aerobic, reduces nitrates, gives a negative Voges-Proskauer reaction and produces acid but no gas in

dextrose, levulose, galactose, maltose, mannitol and sucrose. In litmus milk it produces alkali at the top and acid at the bottom with no coagulation but slow peptonization.

8. Prepare smears of flakes of mucus from a stool and stain by Gram and with carbolfuchsin. If a large number of typical gram-negative, comma-shaped organisms are present, examine a hanging drop preparation. If typical, actively motile vibrios are present, a tentative diagnosis may be made. These procedures are of value in the examination of carriers.

9. Inoculate 2 tubes of alkaline peptone water and prepare plates of Dieu-donne's blood agar. Incubate at 37° C.

10. At the end of 8 to 16 hours examine a hanging drop of a peptone water culture and also prepare a smear stained by the Gram method. To 1 tube add 3 to 5 drops of concentrated sulphuric acid as the cholera vibrio gives a pink reaction (cholera red reaction).

11. With the second tube or with suspicious colonies conduct agglutination tests as follows:

(a) Deposit near one end of a slide a drop of agglutinating serum of a dilution of 1:200 (titer not less than 1:4000) and near the other end a drop of saline. Now touch the suspected colony with the point of the inoculating needle, rub up in the drop of saline solution, then flame the point and again touch the colony with the point and rub up in the drop of serum solution. Evidence of agglutination will almost instantly be apparent in the latter (if cholera). The drops may be allowed to dry and may be fixed and stained. If agglutination has taken place, it will be evident in the stained specimen to the naked eye, or on slight magnification with the hand lens.

(b) If clumping does not occur, test at least 10 (and preferably 20 or 25) such colonies, and examine the preparation, after standing, with the microscope for vibrios.

(c) If evidence of agglutination on the slide is obtained, or in the event that no agglutination takes place, but that the stained preparation shows a vibrio, the colony must be fished and a plain agar slant inoculated for further study.

(d) The crucial test of the specificity of a vibrio is the agglutination test with a serum of high titer.

After incubating the agar slants inoculated with the suspicious colony, or preferably 2 colonies, for 16 to 24 hours, sufficient culture is on hand for an accurate macroscopic agglutination test in a graded series of serum dilutions.

To be cholera it must agglutinate in a dilution of at least 1:1000 (with a serum having a titer of at least 1:4000) within 2 hours at 37° C.

If a vibrio is isolated that fails to do this, repeated daily subculture on agar must be made and its agglutinability tested, for observations are on record showing that



FIG. 196.—*VIBRIO CHOLERAE*

(After Frankel and Pfeiffer.)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)



occasionally (though rarely) a freshly isolated vibrio may show little or no agglutinability but gain it after a series of subcultures.

12. If no suspicious colonies are found on the plates, make plates from the subcultures which are now 14 hours old. This series of plates is examined, after incubating at least 14 hours, in a manner precisely like that prescribed for the original set.

If under these circumstances no suspicious colonies should be found, the examination must be regarded as negative.

In this case the procedure will have extended over a period of about 36 hours.

**Animal Inoculation Test for the Cholera Vibrio.**—Emulsify a loopful of culture from an agar slant in 1 cc. of broth, and inject a guinea-pig intraperitoneally. A fatal peritonitis usually follows within 24 hours.

**The Pfeiffer Bacteriolysis Test.**—1. To 1 cc. of 1:1000 high-titer cholera antiserum in broth add a loopful of 18- to 24-hour agar culture and emulsify.

2. Inject into a guinea-pig intraperitoneally.

3. Inject a second animal with a similar emulsion in 1 cc. of 1:100 normal serum.

4. At intervals of 5, 20, 40 and 60 minutes remove peritoneal exudate with sharp capillary pipets from each animal and examine microscopically as hanging drop preparations followed by smears stained with carbolfuchsin.

5. A positive reaction is indicated by the vibrios losing motility, swelling and undergoing granular degeneration in the pig inoculated with antiserum.

**Method for the Examination of Water for the Cholera Vibrio.**—1. Place 100 cc. in a sterile flask and add 10 cc. of a sterile 10 per cent solution of peptone in water.

2. Incubate at 37° for 12 hours. Transfer a portion of the surface growth to tubes of alkaline peptone water and prepare plates of plain or Dieudonne's blood agar.

3. If suspicious colonies develop identify by methods described above.

### METHODS FOR THE IDENTIFICATION OF *L. ACIDOPHILUS*

1. The *Lactobacillus acidophilus* occurs in the mouth and feces and is believed to be an important factor in the production of dental caries.

2. Materials submitted for examination are usually the feces of individuals taking acidophilus milk and cultures of necrotic teeth.

3. The organism occurs as gram-positive rods, single or in chains and occasionally filamentous.

4. Colonies on tomato juice peptone agar or casein digest agar, are usually large and rough or woolly but may be smooth and round.

5. Litmus milk is coagulated and acidified.

6. Maltose, sucrose, lactose and raffinose are almost always fermented; mannitol rarely and salcin by about one-half of the cultures.

### METHODS FOR THE LABORATORY DIAGNOSIS OF HEMORRHAGIC SEPTICEMIA OF THE LOWER ANIMALS

1. *Pasteurella bovisseptica* (*hollingeri*) causes hemorrhagic septicemia in cattle; *Pasteurella equiseptica* in horses; *Pasteurella suilla* in hogs; *Pasteurella oviseptica* in sheep and *Pasteurella aviseptica* in fowls (fowl cholera).

2. Blood cultures, blood smears on slides, tissues and exudates of edematous swellings, lymphatic glands, spleen and other organs should be examined.

3. Prepare smears and stain with carbolfuchsin. Use Wright's stain for smears of blood, spleen and kidneys.

4. Prepare cultures on plates of blood agar; also on eosin-methylene blue agar for *B. coli* which at times are present and apparently pathogenic.

5. Incubate at 37° C. for 48 hours and examine; prepare smears and stain by Gram method and methylene blue.

6. The organisms occur as small ovoid rods, rounded ends, occurring singly, in pairs or in small bundles.

7. They are nonmotile; noncapsulated in cultures; nonsporulating; gram-negative and bipolar.

8. The colonies are small, round, amorphous, translucent, grayish-yellow to bluish and buttery with no hemolysis but browned.

9. Agglutination tests with specific sera may be employed according to titer although group reactions occur.

10. Inject 1 cc. of broth culture or tissue emulsion into rabbits intravenously or mice intraperitoneally. If material is grossly contaminated inject subcutaneously. Death usually occurs within 1 to 4 days with slightly swollen spleen, petechial hemorrhages of the serous membranes and laryngotracheitis. Prepare cultures of heart blood, spleen, kidneys, peritoneal fluid and lymphatic glands.

#### METHODS FOR THE IDENTIFICATION OF *CLOSTRIDIUM CHAUVOEI* (BLACKLEG)

1. The bacillus of symptomatic anthrax (*Clostridium chauvoei*) is pathogenic for cattle, sheep and goats but the disease is usually confined to cattle and is known as "blackleg".

2. Prepare smears of the exudates and stain by the Gram method.

3. Prepare cultures on dextrose blood agar plates and in liver broth. Cultivate anaerobically as the organism is strictly anaerobic. The colonies are circular with a slightly granular compact center, from which a thinner peripheral zone emanates made up of a tangle of fine threads.

4. The bacillus is a large, motile, sporulating rod with rounded ends (Fig. 197). The spores may be located either near the end or centrally and those showing end spores are spoon-shaped. When freshly isolated from tissue (in contradistinction to anthrax) the organisms show the presence of spores.

5. The organism ferments dextrose, maltose, lactose and saccharose with the production of acid and gas. Mannite and salicin are not fermented. Indol is not produced.

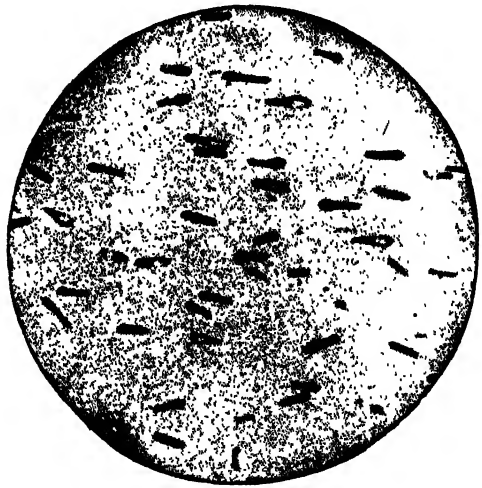


FIG. 197.—*CLOSTRIDIUM CHAUVOEI*

(After Zettnow.)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

6. Inject the hind leg of a guinea-pig with 1 cc. of culture or an extract of tissue. Death usually occurs in 18 to 36 hours with emphysematous gangrene, giving off an odor like that of butyric acid. Examine stained smears for the sporulating bacilli.

### METHODS FOR THE IDENTIFICATION OF *A. NECROPHORUS*

1. *Actinomyces necrophorus* produces a variety of conditions collectively referred to as "necrobacillosis" including foot rot, necrotic stomatitis of swine, calf diphtheria, liver abscesses in cattle, sheep and hogs, gangrenous dermatitis and secondarily in the intestinal ulcers of hog cholera and in canker of the foot in the horse. A few human infections have occurred with the production of localized vesicular and gangrenous dermatitis and possible puerperal infections.

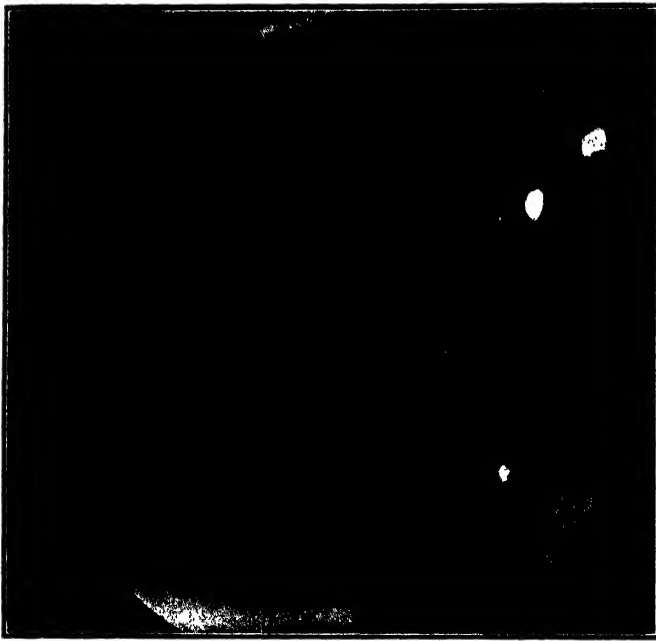


FIG. 198.—*ACTINOMYCES NECROPHORUS*

(Courtesy of Dr. Frederick W. Shaw.)

2. The organism is a strict anaerobe and very difficult to cultivate. If smears of necrotic material show numerous slender, gram-negative beaded filaments measuring 100 microns or more in length which fail to grow aerobically, the diagnosis is usually justified (Fig. 198).

3. Inject a rabbit subcutaneously in the median abdominal line with finely divided tissue and pus. A local necrosis is produced from which the organism may be recovered in large numbers.

### METHODS FOR THE IDENTIFICATION OF *ERYSIPELOTHRIX RHUSIOPATHIAE*

1. Erysipeloid (Rosenbach) of man and "swine erysipelas" are caused by *Erysipelothrix rhusiopathiae* (Kitt).
2. Veterinarians are sometimes infected by handling diseased swine and in the United States the disease is common among handlers of fish (Klauder and Harkins).
3. In human beings the acute septicemic type is rare; infection usually occurs about the fingers and is variously regarded as "fish poisoning" or a pyogenic skin infection. Polyarthritis may occur (Klauder).
4. Excise pieces of skin about 3 millimeters square from infected areas; grind in a sterile mortar and inoculate tubes of hormone broth as the organism grows on ordinary media. Incubate aerobically at 37° C.
5. The organism occurs as a small, slender, gram-positive rod; singly, and in chains; sometimes branching; often granular; nonmotile; noncapsulated; nonsporulating. Old cultures are thread-like.
6. On agar the colonies are small, colorless, translucent, moist and homogeneous; poor growth in broth; hemolysis on blood agar plates; ferments dextrose and lactose.
7. Inoculate mice intraperitoneally or pigeons intramuscularly with 0.2 cc. of 2 to 4 day cultures; usually fatal and pure cultures may be obtained from the blood of the heart by plating on blood agar.
8. Conduct agglutination and complement fixation tests with swine immune serum. Immune serum also gives a precipitin reaction with filtrates of broth cultures and according to Ascoli, also with extracts of infected tissues.

### METHODS FOR THE IDENTIFICATION OF *B. BOTULINUS*

1. In addition to staphylococci and Salmonella, food poisoning may be caused by *B. botulinus* (*Clostridium botulinum*). It is usually due to the ingestion of the preformed exotoxin in preserved vegetables. Consequently, examinations of the stools for the bacillus are not ordinarily required, but may be conducted.
2. In this case anaerobic cultures are required. Inoculate 2 tubes of Brewer's sodium thioglycollate broth; heat one at 70° C. for 20 minutes and then prepare plates of blood agar by the surface streak method. Incubate both tubes and the plates anaerobically at 35 to 70° C. Prepare smears and stain by the method of Gram.
3. *B. botulinus* occurs as a large gram-positive rod with rounded ends, singly or in short chains. Spores are oval, larger than the bacilli, and usually at or near the ends. They form best in sugar-free media at 20 to 25° C.
4. The bacilli are motile and noncapsulated.
5. On blood agar the colonies are irregularly round, umbonate, smooth centers with fimbriate margins and alpha type of hemolysis.
6. In cooked brain broth there is abundant growth with gas and butyric acid odor; the brain is digested and blackened.
7. Type A produces acid and gas in glucose, maltose and salicin; types B and C do not ferment salicin. Types A and B ferment glycerol; type C does not.
8. Test the food for the toxin of *B. botulinus* as follows: (a) Prepare a suspension or dilution of the food (usually the juice in canned vegetables) in sterile

saline solution and centrifuge. (b) Give each of 4 guinea-pigs about 1 cc. by subcutaneous injection. (c) At the same time inject one intraperitoneally with 1 to 1.5 cc. of Type A antitoxin; a second with a like amount of Type B antitoxin and a third the same amounts of both the A and B antitoxins. The fourth pig is a control. If the toxin is present the results will be somewhat as follows:

TABLE 22

Guinea-pigs Protected with Antitoxin	With A Toxin	With B Toxin	With A and B
Pig 1 (Type A).....	Live	Die	Die
Pig 2 (Type B).....	Die	Live	Die
Pig 3 (Types A and B).....	Live	Live	Live
Pig 4 (none).....	Die	Die	Die

9. A similar test can be made with anaerobic broth cultures.

METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF PLAUT-VINCENT'S ANGINA AND FUSOSPIROCHETAL GINGIVITIS

Plaut-Vincent's angina is caused by *Spirochaeta vincentii* (*Borrelia vincentii*) in symbiosis with *Bacillus fusiformis*. These organisms also produce a type of gingivitis commonly called "trench mouth" as well as stomatitis and ulcerating lesions in other parts of the body.

**Plaut-Vincent's Angina.**—1. Smears are required for diagnostic purposes as the organisms are anaerobic and difficult to cultivate. These should be made on glass slides

and not too thin. Swabs accompanying cultures on Löffler's blood serum for diphtheria bacilli may be used for preparing smears and this is a good routine practice as Plaut-Vincent's angina may be mistaken clinically for diphtheria.

2. Dry in air.

3. Fix by passing through flame 4 times.

4. Cover with carbolfuchsin diluted 1:10 with water; heat gently and stain for 2 minutes. Stain second slide by method of Gram.

5. Wash in water and dry.

6. Examine with oil-immersion lens for fusiform bacilli and spi-

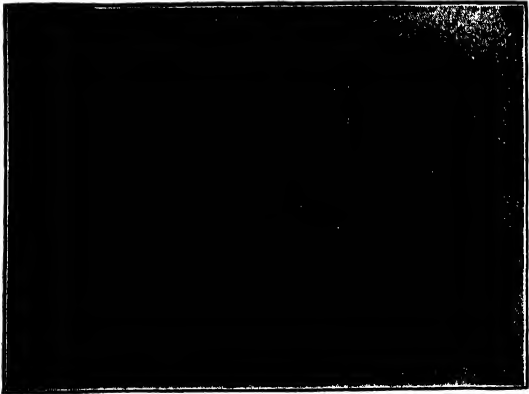


FIG. 199.—FUSIFORM BACILLI AND SPIRILLA OF PLAUT-VINCENT'S ANGINA

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

rilla. The former are gram-negative (variable), long, slightly curved with pointed ends and showing faintly staining granules. The latter are large, wavy spirals (Fig. 199).

7. Both organisms are also readily seen in wet preparations with high dry or oil-immersion objectives or by darkfield examination.

**Fusospirochetal and Amebic Gingivitis.**—1. Wet and stained preparations are recommended.

2. The material should be collected with care, especially from gingival pockets with suitable instruments or after expression by pressure.

3. Prepare several smears. Dry in the air. Fix with heat. Stain with 1 : 10 carbol-fuchsin for 2 or 3 minutes. Wash with water, dry, and examine with oil-immersion lens for spirochetes (*Spirochaeta microdentium*; *Spirochaeta macrodentium*, etc.).

4. An occasional spirochete is normal, but large numbers and tangled masses represent a pathological increase (spirochetic gingivitis).

5. Fusiform bacilli may also be found in association with spirochetes (*Spirochaeta vincentii*) constituting *spirofusillar gingivitis*, a form of Vincent's angina infection of the gums, or trench mouth.

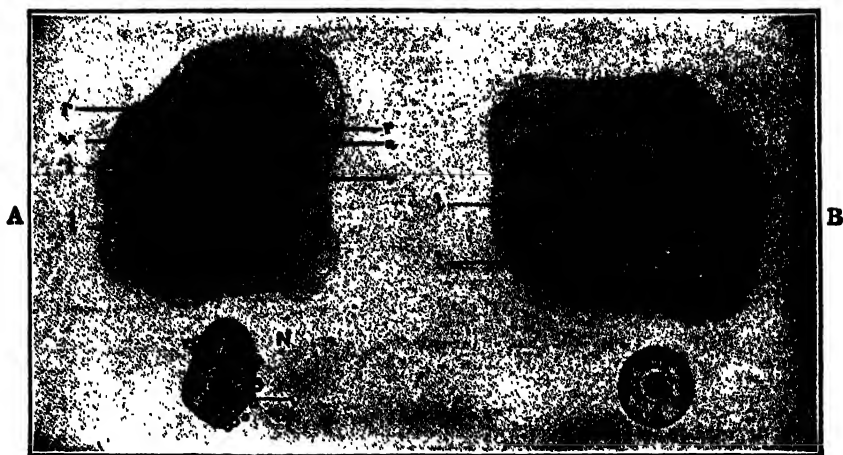


FIG. 200.—ENDAMOEBA GINGIVALIS

A, trophozoite stained with Wright's stain to show characteristic globules (*v*) distributed near nucleus in condensed areas of cytoplasm. B, a similar form stained by the Heidenhain's iron hematoxylin method which fails to show the perinuclear structures. N, nuclear detail at higher magnification. l, ingested leukocyte fragments. n, nucleus. r, ingested red cell fragments. v, perinuclear globules or "vacuoles." (Original drawings by Uribe.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

6. A few spirochetes and fusiform-shaped bacilli resembling *B. fusiformis* are to be found in most mouths and do not alone constitute evidences of infection; but the presence of large numbers is regarded as pathological.

7. *Leptotrichia buccalis* is frequently found in the mouth and may be mistaken for fusiform bacilli. They occur as long, gram-positive bacilli or filaments. They do not grow under ordinary aerobic conditions.

8. Wet preparations are especially desired for examination for *Endamoeba gingivalis* (Gros) which resemble *E. histolytica* (Fig. 200).

Warm a slide. Place a drop of warm saline solution. Add a small amount of gingival secretion. Cover with coverglass and examine for motile amebae with high dry lens and with the light well reduced.

Spirochetes may be likewise detected in these preparations or by darkfield examination.

An occasional ameba may be found in the absence of gingivitis. One or more per field, however, represents an increase and may produce gingival infection by opening up avenues of bacterial infection.

### METHODS FOR THE LABORATORY DIAGNOSIS OF RECURRENT FEVER

1. *Borrelia recurrentis* (*Spirochaeta obermeiereri*) and *Borrelia novyi* (*Spirochaeta novyi*) may be found in blood films fixed with methyl alcohol and stained with diluted carbolfuchsin or after staining with Jenner's or Wright's blood stains (Fig. 201). Careful search for the spirochetes is usually required.



FIG. 201 —BORRELLIA RECURRENTIS (After Calkins)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D Appleton-Century Co, New York.)

2. Cultures are not employed.

3. Inject 0.2 to 0.5 cc. of patient's blood or clots broken up in sterile saline solution intraperitoneally into a white mouse under aseptic precautions.

4. Examine a drop of blood from the tail on a slide covered with cover-glass with high dry or oil-immersion lenses each day over a period of at least 5 to 14 days for spirochetes. Infected mice are likely to survive for months with recurrent spirochetemia.

### METHODS FOR THE IDENTIFICATION OF LEPTOSPIRA

1. Examine for *Leptospira icterohaemorrhagiae* and *Leptospira canicola* in the urine and blood.

2. Urine may be centrifuged and the sediment examined by darkfield method.

3. Catheterized urine is suitable for cultures. Inoculate tubes of the rabbit-serum medium with several loopsful of sediment and cultivate anaerobically.

4. Inject white guinea-pigs (8 to 10 ounces) intraperitoneally with sediment suspended in saline solution (same technic as inoculation for tubercle bacilli).

5. Inoculate white guinea-pigs of same weight intraperitoneally with 0.5 to 1 cc. of citrated blood or with clots broken up in sterile saline solution, taken during the first week of the disease.

6. Inoculate rabbit-serum medium with 0.5 cc. of blood; cultivate anaerobically.

7. Incubate all cultures at 25° C. and examine each weekly by darkfield for at least 4 weeks for motile leptospirae.

8. Examine the guinea-pigs daily for jaundice of the skin and sclerae. Take temperature daily. When a marked rise occurs, remove 1 or 2 cc. of blood from the

heart in an equal amount of 1 per cent citrate solution. Examine by darkfield for leptospirae. If organisms are found, chloroform the animal. Look for jaundiced tissues and numerous petechial (butterfly) hemorrhages, especially in the lungs and inguinal region.

9. Prepare sections of the liver and kidneys to be stained for leptospirae.

**Agglutination and Lysis.**—1. If a culture is available, prepare equal parts of 0.5 cc. of culture and 1 : 2, 1 : 4, 1 : 8, 1 : 16, and 1 : 32 dilutions of patient's serum in small test tubes. Place in water bath for 2 hours. Examine each and a control for agglutination by darkfield.

2. In a test tube place 0.5 cc. of culture (upper portion), 0.5 cc. of the patient's fresh unheated serum and 1.5 cc. of saline solution. Mix and inject the whole into the peritoneal cavity of guinea-pig. At intervals of 15 minutes withdraw a small amount with fine capillary tubes and examine by darkfield for evidences of agglutination and lysis.

3. Inject a second pig with a control mixture, using normal human serum.

4. If antibodies are present, agglutination with partial or complete lysis usually occurs within an hour.

## METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF SYPHILIS

1. *Spirochaeta pallida* (*Treponema pallidum*) may be found in primary and secondary lesions by darkfield examination (recommended) or by special staining methods (Fig. 202).

2. Cultures are not employed.

3. Secretions, bits of tissue, spinal fluid and material aspirated from enlarged lymph glands may be inoculated into the testicles of rabbits. Full-grown and healthy animals should be employed. From 0.2 to 1.0 cc. of fluid or emulsion should be injected into the center of each testicle with a sterile syringe after sterilization of the skin with iodine. Syphilitic orchitis develops in 3 to 6 weeks with numerous spirochetes to be seen by darkfield examination. Chancres of the scrotal skin may occur.

4. According to Loveman and Morris (*Am. Jour. Syph., Gonorr. and Ven. Dis.*, 28: 44, 1944), darkfield examinations for *T. pallidum* of material aspirated from enlarged lymph glands are of particular diagnostic value intraurethral chancres, lesions obscured by phimosis or paraphimosis, old involuted chancres upon which the local darkfield examinations are repeatedly negative, dirty, painful and secondarily infected chancres, all chancres or secondary lesions within the oral cavity (especially chancres of the tonsils), secondary syphilitic lesions with moderate lymphadenopathy, and in the differential diagnosis of syphilis and lymphopathia venereum. The technic of aspiration is as follows: (a) apply tincture of iodine to the skin overlying an enlarged gland; (b) using a 20 to 22 gauge needle, draw up about 0.5 cc. of sterile distilled water or saline solution into a 5 or



FIG. 202.—SPIROCHAETA PALLIDA; LEVADITI METHOD  
(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)



10 cc. Luer syringe; (c) fix a suitable gland with the fingers and insert the needle; (d) when it is certain that the gland has been entered, inject the water; (e) rotate the needle for 30 or 40 seconds and gently move the gland from side to side by moving the needle; (f) withdraw the slightly blood-tinged serum and make a thorough dark-field examination for spirochetes. Positive results are stated to indicate the presence of *T. pallidum* as nonpathogenic spirochetes do not occur in the lymphatic glands.

### METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF RAT-BITE FEVER

1. Rat-bite fever is primarily a disease of wild rats transmissible to man, rats and other animals by bites.
2. The causative organism is *Spirochaeta morsus minus* (*Spirillum minus*).
3. Inoculate white mice or guinea-pigs intraperitoneally with the patient's blood (citrated), exudate from the initial lesion, serum expressed from erythematous patches, material aspirated from lymphatic glands, or ground-up tissue. Examine wet preparations of the blood daily for the organism (Fig. 203). If found, prepare mixtures of

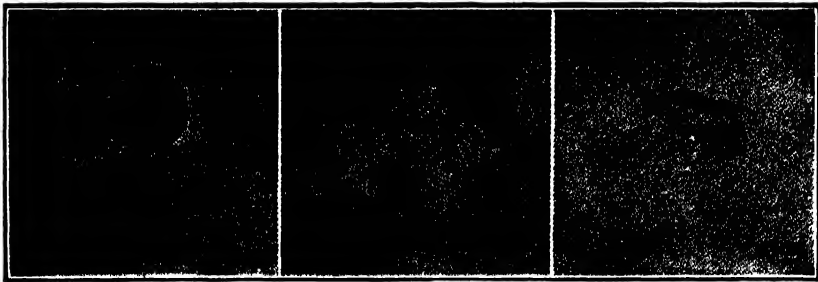


FIG. 203.—SPIROCHAETA MORSUS MURIS

1, in blood of guinea-pig. Short form. Wright's stain. ( $\times 1500$ .) 2, in blood of white mouse. Long form. (Army Med. Museum 50281, from Francis.) 3, in blood plasma of guinea pig. Flagellum at each end stained by Fontana-Tribondeau silver method. (Army Med. Museum 50417, from Francis.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

patient's serum with the blood for loss of motility of the organism. This is a confirmatory test but is often negative, uncertain and subject to error.

4. Examine wet preparations of the patient's blood by darkfield examination although the organism is rarely detected with certainty by this means and there is likelihood of mistaking "artifact spirochetes" for the actual organism. Make similar examinations of secretions from the initial lesion, which are much more likely to be positive.

5. Smears may be stained with the Wright or Giemsa stains.
6. Cultures are not required.

### METHODS FOR THE BACTERIOLOGICAL DIAGNOSIS OF GRANULOMA INGUINALE

1. Granuloma inguinale, granuloma venereum, or groin ulceration is characterized by a swelling which may involve the external genitals, inner surface of the thighs, perineum and anus with involvement of the inguinal lymphatic glands. It occurs more frequently in women.

2. The disease should not be mistaken for lymphopathiae venereum, a disease caused by a filtrable virus, for which the Frei intradermal test is employed for diagnostic purposes.

3. The etiology of granuloma inguinale is uncertain but is probably caused by *Klebsiella granulomatis* (*B. granuloma*), a Friedländer-like organism described by Walker (Fig. 204).



FIG. 204.—KLEBSIELLA GRANULOMATIS OF GRANULOMA INGUINALE

4. The organism occurs in endothelial cells as small oval bodies resembling the "Donovan bodies" of kala-azar.

5. Carefully cleanse the surface of the ulcer with saline solution to remove the pus and then scrape the surface with the edge of a scalpel to obtain tissue cells. Blood and pus should be avoided as much as possible. As the procedure may be painful, it may be necessary to anesthetize the surface with a few drops of novocain or cocaine solution. The scrapings should be deep enough to include many tissue cells as superficial scrapings are usually unsatisfactory.

6. Stain the smears with Wright's or Giemsa stains and examine the cells for the intracellular bodies. Mortara and Dienst (*Jour. Lab. and Clin. Med.* 27: 296, 1943) have recently described the following method: (a) Fix the smear by drying in the air or by gentle heating; (b) flood with 0.5 per cent aqueous solution of basic fuchsin for 2 minutes; (c) wash off excess dye with water and decolorize with an 0.5 per

cent aqueous solution of citric acid until the dye ceases to leave the smear (approximately 5 seconds); (*d*) wash in water and counterstain with a 1 per cent aqueous solution of aniline blue (C.I. No. 707) for 1 minute; (*e*) wash with water, dry, and examine. The intracellular organisms are stained a deep pink and the endothelial cells a deep blue.

7. If cultures are prepared, use Sabouraud's agar on which it occurs as a short plump cocco-bacillus, capsulated, non-motile, non-branching, non-sporulating and gram-negative. Does not liquefy gelatin or produce indol. The colonies are white, moist, convex and viscid.

8. The organism is fatal for guinea-pigs inoculated intraperitoneally with 1 cc. of culture. Subcutaneous or intracutaneous inoculation of mice and rabbits produce local lesions.

According to Pund and Greenblatt (*Arch. Path.*, 23: 224, 1937) granuloma inguinale reveals in the pure or unmixed cases a uniform histologic picture. The essential features are (*a*) the massiveness of the cellular reaction in which the luxuriant granulation tissue is surcharged with plasma cells, (*b*) the relative and conspicuous paucity of lymphocytes, (*c*) the diffuse sprinkling of polymorphonuclear leukocytes, with focal collections in the superficies and papillae, (*d*) the pronounced marginal epithelial proliferation simulating early epitheliomatous changes, (*e*) the pathognomonic large mononuclear cells scattered in various numbers throughout the granulation tissue. The latter are regarded as specific for granuloma inguinale. The relatively large size of the cell, the diameter of which varies from 25 to 90 microns, and the many intracytoplasmic cysts filled with deeply stained bodies are its cardinal features. Donovan bodies are round or rod-like, are grouped within the cysts and have an affinity for hematoxylin. Their recognition is of paramount importance because it permits the diagnosis of granuloma inguinale to be made by histologic study of the tissue.

The affinity of the intracystic bodies for silver salts facilitates the recognition of the characteristic cell. With silver these bodies are stained black to brown and have a closed safety pin appearance due to their elongated ovoid and intense bipolar staining reaction (*Jour. A.M.A.*, 108: 1401, 1937).

## METHOD FOR TESTING THE SUSCEPTIBILITY OF BACTERIA TO PENICILLIN

It is sometimes necessary or advisable to test cultures of microorganisms recovered from patients for susceptibility to penicillin and especially when natural or acquired resistance to penicillin is suspected. The following method may be employed:

1. If the microorganism to be tested is a staphylococcus cultivate in plain beef extract broth at 37° C. for about 6 hours and prepare a 1:100,000 dilution with sterile broth. In the case of cultures of streptococci, pneumococci, meningococci, etc., cultivate in rabbit blood broth (3 cc. sterile rabbit blood per 100 cc. veal infusion broth) for 18 hours and prepare 1:100 or 1:1000 dilutions depending upon the degree of growth.

2. For testing staphylococci place 0.5 cc. of sterile plain broth in each of 10 small sterile test tubes (13 x 100 mm.); for testing streptococci, pneumococci, etc., place 0.5 cc. of rabbit blood broth in each tube.

3. Prepare a solution of penicillin in cold sterile plain broth to carry 10 units per cc.

4. Add 0.5 cc. of the penicillin solution to tube No. 1; mix and transfer 0.5 cc. to tube No. 2 and so on to tube No. 9 from which discard 0.5 cc. after mixing. The tubes now carry 5 units of penicillin (No. 1) to 0.02 units (No. 9) respectively. Tube No. 10 receives no penicillin and is the culture control.

5. Add 0.5 cc. of diluted culture to each of the 10 tubes. Mix well and incubate at 37° C. for 18 to 24 hours.

6. The culture control should show a good growth. In the case of a penicillin sensitive microorganism growth is usually inhibited in the first 8 or 9 tubes. In the case of a penicillin-resistant microorganism growth occurs in some or all of the first 9 tubes depending upon the degree of resistance.

## METHODS FOR ASSAYING PENICILLIN IN THE BODY FLUIDS

A number of methods have been proposed for the detection and assaying of penicillin in the blood, plasma, serum, spinal fluid, urine, exudates (bronchial, pleural, synovial, etc.) and other body fluids in relation to its dosage and routes of administration in the treatment of disease. At the present time, however, there are no chemical methods for these purposes; all are of necessity microbiological in character and based upon the degree of bacteriostatic or bactericidal activity of penicillin in these materials in vitro for penicillin-sensitive microorganisms as compared with solutions of a standard penicillin of known unitage.

**Collection of Materials.**—Whole blood is not generally employed and especially in the Oxford cup method because the sedimentation of erythrocytes interferes with the diffusion of penicillin from the cups into the agar medium. Serum or plasma is generally used, especially the former, and is stated to carry about 90 per cent of the penicillin in the blood. About 5 cc. of blood should be collected aseptically at hourly or half-hourly intervals over a period of 4 hours following the injection of penicillin or at any other chosen interval or intervals. If serum is to be tested each specimen should be placed in a sterile test tube for coagulation and the separation of serum. If whole blood or plasma is to be tested place 5 cc. of blood in a test tube carrying 0.8 cc. of a sterile 2.5 per cent solution of chemically pure sodium citrate evaporated to dryness in the tube for the prevention of coagulation. It is advisable to centrifuge the serum or plasma, if necessary, for the removal of erythrocytes. In the case of spinal

fluids and exudates the method of collection should be the same as for whole blood or plasma when coagulation is expected to occur. *All specimens should be immediately placed in a refrigerator at 0 to 5° C. and kept at this temperature until tested to preserve the penicillin.*

**Glassware.**—All glassware should be cleaned by means of chemicals and sterilized with dry heat.

**Standard Penicillin.**—Penicillin assays require that a standard penicillin of known unitage be tested side by side with the unknowns and the values of the latter calculated from a standard curve. Small amounts of dry standard penicillin may be obtained from manufacturing laboratories.

The dry standard and solutions prepared from it whose unitage has been carefully determined directly or indirectly against the original Oxford standard, should be stored in a refrigerator at all times. The container of dried standard penicillin should be allowed to come to room temperature in a desiccator before opening. For a stock solution, 2 to 10 mg. are accurately weighed as rapidly as possible on an analytical balance. The stock solution is made to contain 32 Oxford units per cc. with sterile M/50 phosphate buffer at pH 7.0. It is best to keep this stock solution frozen because at ordinary refrigerator temperatures it tends to slowly lose activity over a period of a few weeks. For use 0.5 cc. of this stock solution is diluted with 7.5 cc. buffer solution to give 2 units per cc. Subsequent dilutions to give the desired final concentrations used for making up the standard curve may be prepared as follows:

Solution containing 2 units per cc. cc.	Buffer cc.	Final concentration (units per cc.)
2.0	0	2.0
1.6	0.4	1.6
1.2	0.8	1.2
0.8	1.2	0.8
0.5	1.5	0.5
0.2	1.8	0.2

*All standard solutions should be kept cold to within a few minutes before use.*

**Oxford Cup Method.**—This method is based upon that of Abraham *et al* (*Lancet*, 2: 177, 1941). Under proper technical conditions it possesses an accuracy near  $\pm 15$  per cent. In principle it depends upon the diffusion of penicillin from material placed in cups in agar plates seeded with the test microorganism. Any penicillin-sensitive culture of *Staphylococcus aureus* may be employed but it is advisable to use the Heatley strain designated as *Staphylococcus aureus* H. The diffusion of the penicillin into the seeded agar produces clear zones of inhibition of growth around the cups, the diameters of which vary with the amount of penicillin in the material being assayed. It is of particular value whenever exudates or contaminated fluids are being tested since the sterility of specimens is not essential.

1. Cultivate *Staphylococcus aureus* in broth for 18 to 24 hours at 37° C. Melt ordinary nutrient agar (should be clear) and cool to 42-45° C. To each 100 cc. add 0.1 cc. of the broth culture and mix thoroughly. With an open tip pipet transfer 13 cc.

amounts to sterile Petri dishes and allow to harden. Immediately place the dishes in a refrigerator where they can be stored for at least 24 hours before use. Cooling prevents the bacteria from developing too soon and also renders the agar better for placing the cups. The seeded dishes should never be allowed to stand for more than a few minutes at room temperature before being used.

2. The cups are small cylinders made of glass, porcelain or stainless steel (Bondi). Those usually employed are cut uniformly from plain pyrex glass tubing (8 mm. outside diameter; 1 mm. wall thickness) by means of a motor-driven glass-cutting disc. They should be about 12 mm. in length and of uniform size. Perfectly flat edges are desirable; chipped or uneven cylinders should not be used. The cylinders should be sterilized with dry heat in an upright position in Petri dishes (Fig. 204A).



FIG. 204A.—GLASS CYLINDERS USED IN THE OXFORD CUP METHOD  
(Courtesy of Merck & Co., Inc., Rahway, N. J.)

One end of each cylinder is warmed by passing momentarily through a Bunsen flame by means of a forceps and set lightly on the surface of the seeded agar. The cups should not be warm enough to sink into the agar. Properly sealed cups do not permit any free liquid to escape from the cup during the whole incubation period. Inverting the dishes tests whether the cups are securely sealed; they should remain attached. Six cups are placed equidistantly around each dish. After setting the cups each dish should be immediately returned to the refrigerator. Three dishes should be prepared for the standard penicillin and 3 for each specimen to be assayed.

Each cup should be filled almost to the top but extreme accuracy is not essential since minor variations in the amounts of fluid have little or no effect upon the results. A single sterile medicine dropper or pipet may be used by rinsing briefly in 5 per cent phenol followed by sterile distilled water and finally by one rinse with the next solution to be tested. The individual cups need not be marked if a vertical wax pencil mark is made on the side of the Petri dish bottom opposite one of the cups. Beginning with that cup, the others are identified by clockwise sequence.

3. Remove 3 seeded dishes with the cups in position from the refrigerator and fill a cup on each dish with each of 6 dilutions of standard penicillin carrying 0.2 to 2.0 units per cc. Immediately place the dishes in an incubator at 37° C. for at least 18 to 24 hours.

4. If the fluid to be tested is believed to contain less than 1 unit of penicillin per cc. it need not be diluted. Fill a cup with the fluid. Under these circumstances 1 dish with 6 cups suffices for the examination of 6 fluids but 3 dishes are used for greater accuracy. If the fluid to be tested is believed to contain more than 1 unit of penicillin per cc. (which is unlikely in the case of plasma, serum, spinal fluid, exudates, etc.) it should be diluted with M/50 phosphate buffer at pH 7.0 to contain not more than 0.5 to 1.0 Oxford unit per cc. One cup in each of 3 dishes should be filled with the diluted fluid. Incubate the dishes in the same manner as for the standard penicillin.

5. After incubation, different sizes of clear circular zones of inhibition of growth of *Staphylococcus aureus* are observed depending on the concentration of penicillin in the cups (Fig. 204B). Sometimes when *Staphylococcus aureus* H is used the clear

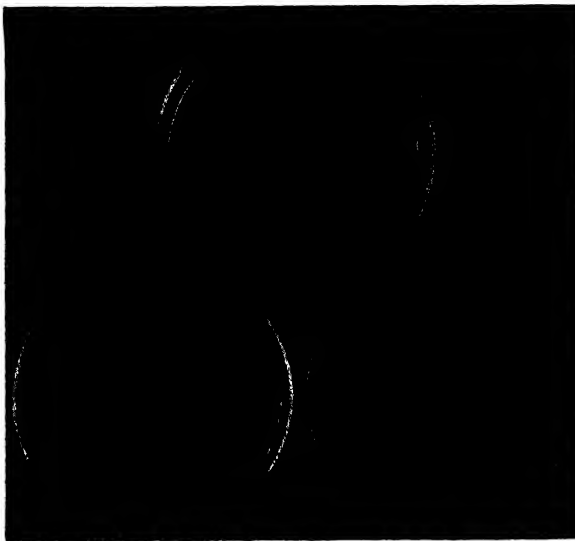


FIG. 204B.—OXFORD CUP ASSAY METHOD

Top plate shows cups on seeded agar ready for incubation. Lower right plate shows inhibition zones for a standard curve after incubation. Lower left plate shows inhibition zones produced by some unknown specimens. (Courtesy of Merck & Co., Inc., Rahway, N. J.)

zone of inhibition around a cup is surrounded by a halo of partial inhibition, which varies from a faint ghost to almost complete inhibition. The cause of this phenomenon is unknown. Each zone should be measured with a millimeter rule within at least one-half millimeter.

6. Calculate the average zone diameters for the corresponding cups on the 3 dishes carrying the 6 dilutions of standard penicillin and prepare a standard curve as shown in Figure 204C.

7. Calculate the average zone diameters for the material being tested and project

from the ordinate horizontally to the standard curve and thence vertically to the abscissa which gives the unitage of penicillin producing the same zone inhibition as the fluid being tested. If the specimen being tested has been diluted multiply by the dilution factor. The result expresses the penicillin content of the specimen being tested in terms of units of penicillin per cc. Greatest reliability of results is obtained if the values for the unknown fall within points on the standard curve corresponding to about 0.3 to 1.4 units per cc.

**Rammelkamp Method.**—In this method (*Proc. Soc. Exptl. Biol. and Med.* 51: 95, 1942) arrange a series of 12 small sterile test tubes and place 0.2 cc. of veal infusion broth in each except No. 1.

2. Place 0.2 cc. of the specimen in Nos. 1 and 2; mix No. 2 and transfer 0.2 cc. to No. 3; mix No. 3 and transfer 0.2 cc. to No. 4 and so on to No. 12 from which discard 0.2 cc. after mixing. The dilutions are now 0, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048 respectively. If the material being tested is known to contain a very small quantity of penicillin, an additional tube carrying 0.5 cc. of the material may be added.

3. Arrange a second series of 12 test tubes and place 0.2 cc. of veal infusion broth in each except No. 1. Prepare a solution of standard penicillin in 0.85 per cent saline solution carrying 20 Oxford units per cc. Place 0.2 cc. in Nos. 1 and 2; mix No. 2 and transfer 0.2 cc. to No. 3; mix No. 3 and transfer 0.2 cc. to No. 4 and so on to No. 12 from which 0.2 cc. is discarded after mixing. The tubes now carry 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039 and 0.00195 units respectively.

4. Cultivate a penicillin-sensitive strain of hemolytic streptococcus belonging to group A in veal infusion broth containing 1 per cent rabbit erythrocytes for 12 hours at 37° C. Dilute with the medium so that the final number of streptococci varies from 1,000 to 10,000 per cc. Add 0.5 cc. to each tube of both series.

5. Set up a culture control of 0.2 cc. of veal infusion broth plus 0.5 cc. of the diluted culture.

6. Incubate all tubes at 37° C. for 18 hours. Streak a 3 mm. loop of the cultures near the endpoint on blood agar plates as a check of sterility.

7. The concentration of penicillin in the specimen being tested is determined by multiplying the highest dilution showing sterility and no hemolysis by the smallest amount of standard penicillin in units showing sterility and no hemolysis as per the following example:

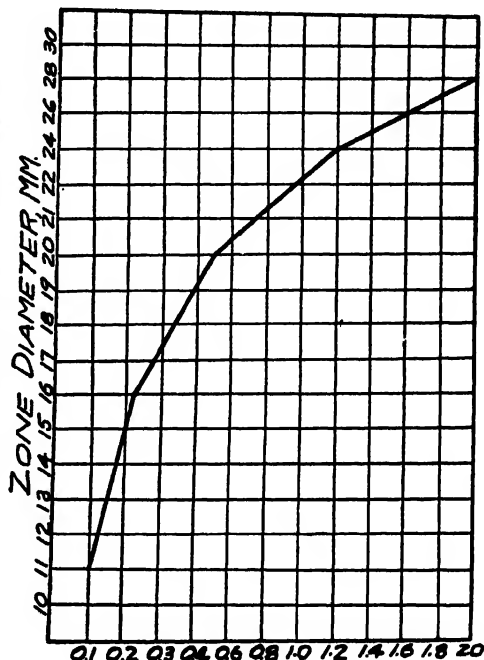


FIG. 204C.—EXAMPLE OF A STANDARD CURVE DERIVED FROM THE OXFORD CUP METHOD



Highest sterile dilution of specimen = 1:64

Smallest sterile amount of penicillin = 0.0039 units

$64 \times 0.0039 = 0.2496$  units penicillin per 1 cc. of specimen

**Kolmer Method.**—1. For each specimen arrange 10 small (13 × 100 mm.) sterile test tubes. Place 1.5 cc. of beef heart infusion broth containing 0.25 per cent glucose in No. 1 and 1 cc. in each of the remaining tubes.

2. Add 0.5 cc. of the specimen being tested to tube No. 1; mix and transfer 1 cc. to No. 2; mix No. 2 and transfer 1 cc. to No. 3 and so on to No. 10 from which 1 cc. is discarded after mixing. The final dilutions are now 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048 respectively.

3. Prepare a solution of standard penicillin in cold saline solution or M/50 phosphate buffer at pH 7.0 carrying 4 units per cc.

4. Set up a second series of 10 small sterile test tubes and place 1.5 cc. of sterile beef heart infusion glucose broth in No. 1 and 1 cc. in each of the remaining tubes. Add 0.5 cc. of the solution of penicillin to tube No. 1; mix and transfer 1 cc. to No. 2; mix No. 2 and transfer 1 cc. to No. 3 and so on to No. 10 from which 1 cc. is discarded after mixing. The tubes now contain 1, 0.5, 0.25, 0.125, 0.0625, 0.031, 0.016, 0.008, 0.004 and 0.002 units respectively.

5. Cultivate *Staphylococcus aureus* H, or any penicillin-sensitive strain of *Staphylococcus aureus*, in broth for 18 to 24 hours at 37° C. Dilute 1:1000 with sterile broth and with a sterile 1 cc. pipet add 1 drop to all tubes of both series.

6. Prepare a culture control of 1 cc. of glucose broth to which is added 1 drop of the diluted culture.

7. Mix and incubate all tubes at 37° C. for 16 to 18 hours.

8. The culture control should show a good growth. Examine the series of tubes carrying the varying amounts of penicillin and record the smallest amount of penicillin in units (highest dilution) showing inhibition of growth. Examine the series of tubes carrying the specimen being tested and record the highest dilution showing inhibition of growth.

9. Multiply the highest inhibiting dilution of the specimen by the smallest inhibiting amount of penicillin in units. The result expresses the approximate amount of penicillin in units per cc. of specimen being tested as per the following example:

Highest inhibiting dilution of specimen = 1:32

Smallest inhibiting amount of penicillin = 0.008 units per cc.

$32 \times 0.008 = 0.256$  units penicillin per 1 cc. of specimen.

**Rake and Jones Method.**—This is a rapid test (*Proc. Soc. Exper. Biol. and Med.*, 54: 189, 1943) for the estimation of penicillin based upon the inhibition of hemolysis of rabbit erythrocytes by beta hemolytic streptococcus; under optimal conditions the results can be read within 55 to 90 minutes. As slightly modified the test may be conducted as follows:

1. Arrange 8 small sterile test tubes (13 x 100 mm.) and place 0.1 cc. of sterile saline solution in each. Add 0.1 cc. of the specimen to No. 1; mix and transfer 0.1 cc. to No. 2 and so on to No. 8 from which discard 0.1 cc. after mixing. The dilutions now range from 1:2 in tube No. 1 to 1:256 in tube No. 8 respectively.

2. Prepare a solution of standard penicillin in saline solution carrying 1 unit

per cc. Arrange a second series of 8 tubes and place 0.1, 0.08, 0.06, 0.04, 0.03, 0.02, 0.015 and 0.01 cc. in the tubes which now carry from 0.1 unit in No. 1 to 0.01 unit in No. 8 respectively.

3. Cultivate a strain of beta hemolytic streptococcus (strain C-203 preferred) in a tube of 1 per cent defibrinated rabbit's blood broth at 37° C. for 2 to 3 hours. Inoculate 6 cc. of the blood broth with 0.3 cc. of the culture and add 0.2 cc. to each tube of both series.

4. As a control of culture place 0.1 cc. of distilled water in a tube and add 0.2 cc. of the inoculated blood broth. As a control of preformed hemolysin place 0.7 cc. of the solution of standard penicillin (0.7 unit) in a tube and add 0.2 cc. of the inoculated blood broth.

5. Mix all tubes and place them in a water bath at 37° C. Make the readings in 55 to 90 minutes.

6. The culture control should show hemolysis; the second control will show only hemolysis from preformed hemolysin (if any) but no additional hemolysis because the growth of the streptococcus is inhibited. In reading the results the first signs of hemolysis can be most readily detected by whirling the tubes slightly and throwing a plume of erythrocytes into the clear supernatant broth. In the presence of a trace of hemolysin this plume becomes rapidly hemolyzed. All tubes showing such commencing hemolysis are thoroughly mixed and replaced in the water bath for 2 or 3 minutes by which time uniform hemolysis will have appeared. In most cases all tubes which are to show hemolysis will do so at almost the same time and an end point is obtained by taking the last tube in each series showing no hemolysis. Occasionally a tube shows hemolysis delayed by about 30 minutes over the others. Such a tube is given a  $\pm$  reading and an interpolated potency.

**Fleming Slide-Cell Method.**—This method is an adaptation of the Wright slide cell technic for measuring the bactericidal activity of defibrinated blood which Fleming (*Lancet*, 1: 732, 1942; *ibid.*, 2: 434, 1943) has recently employed for the detection and assaying of penicillin in the serum of patients under treatment. With slight modifications the technic may be as follows for testing serum or spinal fluid:

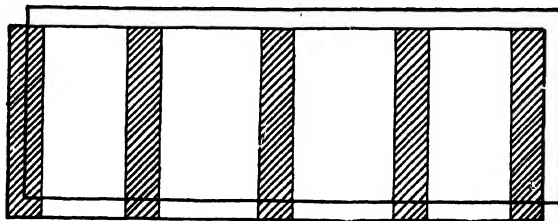


FIG. 204D.—WRIGHT SLIDE CELL

1. Slide cells are prepared as follows: (a) Sterilize an ordinary microscopic slide by passing it through a Bunsen flame; (b) dip five narrow strips of paper (1/12th mm. in thickness) in hot vaseline and lay these down at both ends and at equal intermediate distances along the slide; (c) sterilize a second slide and press it down upon the vaselined strips as shown in Figure 204D.

2. For each specimen arrange 8 small sterile test tubes. Place 1.5 cc. of sterile broth in No. 1 and 1 cc. in each of the remaining tubes. Add 0.5 cc. of the specimen

to No. 1; mix and transfer 1 cc. to No. 2; mix No. 2 and transfer 1 cc. to No. 3 and so on to No. 7 from which 1 cc. is discarded after mixing. The final dilutions are now 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 respectively. Tube No. 8 is the culture control.

3. Prepare a solution of standard penicillin in cold saline solution carrying 1 unit per cc. Set up a second series of 8 small sterile test tubes and place 1.5 cc. of broth in No. 1 and 1 cc. in each of the remaining tubes. Add 0.5 cc. of the solution of penicillin to tube No. 1; mix and transfer 1 cc. to No. 2 and so on to No. 8 from which 1 cc. is discarded after mixing. The tubes now carry 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004 and 0.002 units of penicillin respectively.

4. Cultivate *Staphylococcus aureus* (H strain preferred) in broth for 24 hours and dilute 1:80,000 with sterile broth. With a sterile pipet add 0.05 cc. to all tubes of both series and mix.

5. With a sterile pipet or medicine dropper fill 8 cells of 2 slides with the 7 seeded dilutions of specimen and the culture control. The same pipet or medicine dropper may be used after rinsing briefly with 5 per cent phenol, followed by a wash in sterile distilled water and finally by 1 rinse with the next dilution. When the cells have been filled in, the upper slide is brought back into position and the ends and sides carefully sealed by brushing them over with very hot paraffin.

6. Fill 8 cells in the same manner with the 8 seeded dilutions of standard penicillin.

7. Incubate the slide cells at 37° C. for 18 hours and examine microscopically with a lower power objective for colonies of staphylococci.

8. Multiply the highest inhibiting dilution of the specimen by the smallest amount of penicillin in units showing inhibition. The result expresses the amount of penicillin in units per cc. of serum as per the following example:

Highest inhibiting dilution of serum = 1:32

Smallest inhibiting amount of penicillin = 0.004 units per cc.

$32 \times 0.004 = 0.128$  units of penicillin per 1 cc. of specimen

# METHODS FOR THE PREPARATION OF BACTERIAL VACCINES AND BACTERIOPHAGE

In this section are described methods for the preparation of such autogenous and stock bacterial vaccines or bacterins as may be required of a clinical laboratory. The tuberculin, diphtheria and tetanus toxoids, scarlet fever toxin, etc., may be obtained commercially and are omitted.

## PREPARATION OF CULTURES

1. Freshly isolated organisms are preferred.
2. The method employed for making cultures is very important in order to secure the organism or organisms responsible for infection. Faulty methods may result in securing only saprophytes or contaminating organisms and defeat the purpose of vaccine therapy at the outset.
3. It is particularly important to use the proper medium and especially when infection with fastidious organisms is suspected (streptococci, pneumococci, gonococci, etc.). Blood agar or glucose hormone broth are recommended for routine use. The preliminary examination of a stained smear of pus or other material is advised and aids in choosing the proper medium.
4. Incubate cultures for 24 to 48 hours; examine smears stained by the Gram method.
5. If more than one organism is present, secure each in pure culture by plating. While the vaccine is being prepared, subcultures of each may be subjected to final identification.
6. When an organism shows both smooth and rough colonies, select the former for the vaccine.
7. Cultivate the organism or organisms on slants of solid medium until sufficient growths are secured; or cultivate in a broth medium and centrifuge thoroughly. Discard the supernatant fluid and use the sediment of bacteria.
8. Examine each culture by stained smear for purity.

## SELECTION OF ORGANISMS

1. In mixed infections with two or more organisms a selection must be made of those to be incorporated in autogenous vaccines. Do not use spore-forming bacilli (like *B. subtilis*) or saprophytes (like *B. prodigiosus*, diphtheroids, etc.). Organisms of secondary infection may be included.
2. Intracutaneous tests with individual vaccines of each organism are sometimes employed for aid in selection on the principle that only those yielding positive allergic reactions should be employed as indicative of infection. Great care is required since skin reactions may be purely inflammatory and nonspecific, or due to the presence of toxins for which there are insufficient amounts of antitoxin in the blood. The exact value or status of the method is as yet unknown.

**The Heist-Cohen Pathogen-Selective Method.**—This method may be helpful on the basis that only those organisms capable of surviving and growing in the whole

coagulated blood of the patient are apt to be infective. It is possible, however, that an organism unable to grow in the blood or produce septicemia may still be able to produce local infection.

1. Secure material to be cultured on sterile swabs (from nose, tonsils, extracted teeth, tooth sockets, sputum, pus, etc.), and rub each swab on the bottom of dry sterile tubes into which 1 or 2 drops of broth have been placed.

2. Then place the swabs into tubes of hormone broth medium for controls and also for use in preparing the vaccine.

3. Immediately secure from 10 to 12 cc. of blood from a vein at the elbow of the patient with a sterile syringe and with aseptic precautions.

4. Place 3 to 5 cc. of blood in each tube in which material has been smeared.

5. Incubate the blood and the control tubes for 24 hours. Examine each by smear. If necessary, plate each on blood agar for identification of organisms. Also inoculate a tube of hormone broth from each blood tube showing a growth as a check on the plate and for preparing the vaccine.

6. The vaccine is now made up by mixing 1 part of the original broth culture and 9 parts of the broth subculture of the blood.

7. Count the mixed suspension and finish up the vaccine according to steps 3, 4, 5 and 6 of the Kolmer method described on page 495.

### PREPARATION OF SUSPENSIONS

1. If a solid medium is used, cover the growth on one of the tubes with sterile salt solution, taking precautions against contamination. Bring the organism into suspension either by shaking or with a platinum loop (Fig. 205).

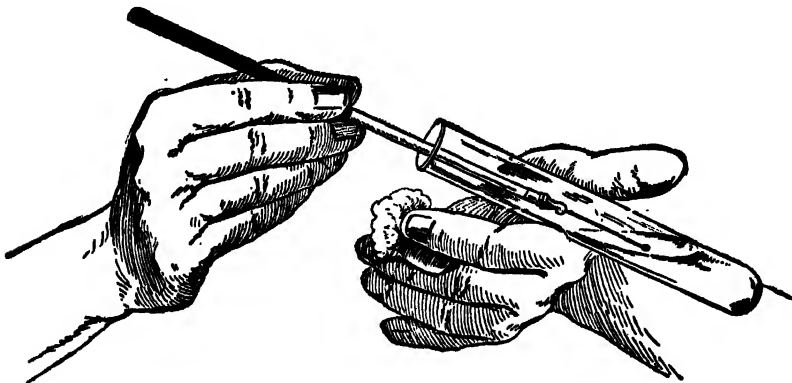


FIG. 205.—PREPARATION OF A BACTERIAL VACCINE

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

2. The suspension from the first tube should be transferred to the second tube and the growth removed in the same manner. Repeat the process until the growths from all tubes have been suspended.

3. If bouillon is used, the culture should be centrifuged at high speed, the bouillon removed and the sediment containing the bacteria resuspended in salt solution.

If the bouillon contains other than human serum, add saline solution to the sediment, mix, centrifuge and resuspend the bacteria in salt solution.

4. The suspension prepared in either manner should be quite heavy.

5. Transfer the suspension to a sterile flask or bottle containing sterile beads and shake well to break up clumps (clumps of staphylococci and typhoid bacilli are easily broken up; streptococci, pneumococci and diphtheroids require more shaking). A mechanical shaker is recommended. The one illustrated in Figure 206 will be found very satisfactory.

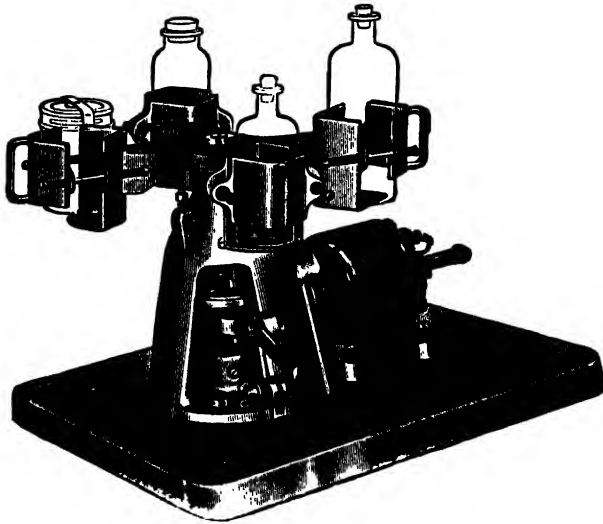


FIG. 206.—MECHANICAL SHAKER FOR VACCINES

6. Filter the suspension through sterile paper (it is convenient to have on hand small funnels with folded paper in place wrapped in newspaper and sterilized in hot air oven).

### STANDARDIZATION OF VACCINES

1. As a general rule, vaccines for administration to adults may contain a total of approximately 1,000,000,000 organisms per cc. For children, one-half this strength may be used.

2. A dose of 0.1 cc. of such vaccines will carry 100,000,000 and 50,000,000 respectively, which are ordinarily satisfactory for the first injections. It is possible, however, to give .05 cc. with a tuberculin syringe if a smaller initial dose is desired. Subsequent doses may be increased by 0.1 or 0.2 cc. as desired.

3. If two or more organisms are to be used, a suspension should be prepared of each containing 1,000,000,000 per cc. (adult). After sterilization, *equal* parts of these individual vaccines may be mixed to make one vaccine.

**Counting Chamber Methods (Recommended).**—1. In a small sterile test tube place 0.1 cc. of suspension and 4.9 cc. of sterile saline solution (gives a 1 : 50 dilution); use sterile pipets. Mix well.

2. Draw the suspension up to the 0.5 mark in a leukocyte-counting pipet.

3. Draw the following stain up to the 11 mark (gives 1 : 20 dilution):

Crystal violet (sat. alc. sol.) ..... 10 cc.

Water (freshly distilled) ..... 100 cc.

Filter a small portion immediately before using.

4. Slip a wide rubber band over the ends of the pipet and shake for at least 2 minutes. Do not allow the dilution to stand.

5. Discard a few drops on a piece of filter paper (discard into a disinfecting solution) and then place a drop in the center of a Helber counting chamber (thoroughly cleaned to avoid dust particles).

6. Accurately adjust a reinforced precision coverglass.

7. Allow to stand for 15 minutes.

8. With a No. 6 objective and No. 4 eyepiece make a count of the bacteria in at least 20 squares, being careful to focus on different levels for bacteria that have not settled.

9. If the number of bacteria is too large for a fairly accurate count (the number per square should be within 10 per cent of each other) repeat, using a 1:100 or 1:200 dilution of vaccine.

10. Divide the total bacteria in 20 squares by 20 to obtain the average per square.

11. Multiply by 400,000,000 to give the number per cc. of dilution:

$$\frac{1}{20} \text{ mm.} \times \frac{1}{20} \text{ mm.} \times \frac{1}{50} \text{ mm.} = \frac{1}{20,000} \text{ c.mm. (contents of a square)}$$

$$\frac{1}{20,000} \times \frac{1}{20} (\text{dilution in pipet}) = \frac{1}{400,000} \text{ c.mm. or } \frac{1}{400,000,000} \text{ cc.}$$

12. Then multiply by 50 (if an original 1:50 dilution was employed) to obtain the number of organisms per cc. of undiluted suspension.

13. Instead of the above, the Petroff-Hauser (Fig. 207) bacteria counter gives excellent results.

With a *red corpuscle* pipet, draw undiluted suspension to the mark 0.5 and stain (same as above) to 101 to give a 1:200 dilution. After thorough agitation, discard a few drops and place a drop into the counting cell. Adjust the coverglass and allow 15 minutes for thorough settling. Make a count of 10 to 20 squares with the following formula:

$$\frac{\text{total bacteria counted} \times 200 \times 20,000,000}{\text{number of small squares counted}} = \text{bacteria per cc.}$$

14. Place the counting chambers and cover slides in 2 per cent cresol for at least 5 minutes before wiping. Clean the pipets in the same before drying.

15. After counting, the vaccine is sterilized and diluted to proper strength as described on page 494.

**Wright's Method.**—1. Make a mark on the stem of a capillary pipet about 1 inch from the tip and fit a rubber bulb to its barrel.

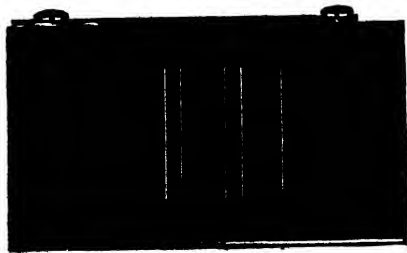


FIG. 207.—THE PETROFF-HAUSER BACTERIA COUNTER

2. Cleanse and prick the finger.

3. Draw up into the capillary pipet sodium citrate solution to the mark on the tip. Then draw a little air in, then blood from the finger up to the mark. Draw a little air in to separate the solution, next draw bacterial suspension up to the mark (Fig. 208).

4. Expel the contents of the pipet on a glass slide or in a watch glass and mix thoroughly by aspirating and re-expelling about a dozen times.

5. Make 2 or 3 thin films on slides in the same manner as described for blood smears.

6. Dry in air and fix with a saturated solution of mercuric chloride.

7. Wash and stain with dilute carbolfuchsin (1:10) or carbol-thionin for 2 to 5 minutes.

8. Wash and dry.

9. Examine only satisfactory films which show bacteria and blood cells in approximately the same numbers and free from bacterial aggregates. With oil-immersion lens count the number of corpuscles and bacteria in a number of fields, or until 500 corpuscles have been counted. Mark down the number of cells and bacteria counted separately and total each at the end.

10. Calculation: Let us assume that 500 red cells and 1000 bacteria have been counted. One c.mm. of blood contains approximately 5,000,000 red corpuscles and equal volumes of blood and emulsion were taken. One c.mm. of the emulsion, therefore, contains

$$\frac{5,000,000 \times 1000}{500} = 10,000,000 \text{ organisms per c.mm., or } 10,000,000,000 \text{ per cc.}$$

11. Dilute to proper strength and sterilize (page 494).

**Nephelometer Method (McFarland).**—1. *This method is mainly applicable for suspensions prepared from agar slants or from centrifuged broth cultures resuspended in saline solution.*

2. Place 1 cc. of bacterial suspension in a test tube which should be of the same size as those used in the nephelometer.

3. Dilute with 4 to 10 cc. of salt solution, keeping accurate record of the final dilution.

4. Compare with tubes of nephelometer. Shake the tubes well before comparing.

5. Calculation: Multiply the number of bacteria represented by the nephelometer tube which corresponds with the density of the bacterial suspension by the dilution of the bacterial suspension. For example, assume that the density of the bacterial suspension corresponds to the No. 3 tube of the nephelometer and before making comparison it was diluted 8 times. No. 3 tube corresponds to 1,000,000,000; 8 times this number equals 8,000,000,000 bacteria per cc. Or simply dilute vaccine to correspond in density to tubes 3 to 4 of the nephelometer to secure approximately 1,000,000,000



FIG. 208.—A CAPILLARY PIPET FOR COUNTING A BACTERIAL VACCINE  
(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)



per cc. for adults; or to correspond to tubes 1 to 2 to secure approximately 500,000,000 per cc. for children.

6. The nephelometer is prepared as follows:

(a) Arrange 10 test tubes or ampules of uniform size in a rack, and label 1 to 10.

(b) Add the following amounts of a 1 per cent aqueous solution of chemically pure barium chloride: To tube No. 1, 0.1 cc.; tube No. 2, 0.2 cc.; and so on, increasing 0.1 cc. in each.

(c) Add sufficient of a 1 per cent chemically pure sulfuric acid solution to make the total volume 10 cc. in each tube.

(d) Seal the tubes or ampules.

(e) When the fine white precipitate of barium sulphate, which has formed in the tubes, is shaken up well, each tube will have a different density, increasing from Nos. 1 to 10. The density of the tubes corresponds *approximately* to bacterial suspensions, as follows:

No. 1: 300,000,000	No. 6: 1,800,000,000
No. 2: 600,000,000	No. 7: 2,100,000,000
No. 3: 900,000,000	No. 8: 2,400,000,000
No. 4: 1,200,000,000	No. 9: 2,700,000,000
No. 5: 1,500,000,000	No. 10: 3,000,000,000

7. If vaccines are prepared of broth cultures, the nephelometer should be prepared with 1 per cent sulfuric acid in broth in order to convey the color of the latter.

8. Since autogenous vaccines are usually made up to carry a total of approximately 1000 million organisms per cubic centimeter, a nephelometer showing this numerical concentration can be prepared of one tube by adding 4 cc. of sterile saline solution to 4 cc. of commercial triple typhoid-paratyphoid vaccine (total 2,000 million per cc.) and sealing in a pyrex glass test tube. For vaccines prepared of broth cultures, the nephelometer may be prepared in the same manner except that sterile broth is used instead of saline solution as a diluent.

## DILUTING, STERILIZING AND PRESERVING VACCINES

**Chemical Sterilization.**—1. Vaccines sterilized with tricresol, cresol, merthiolate or phenol without the aid of heat are commonly regarded as being more antigenic than heat-killed vaccines.

2. Tricresol is recommended in a final concentration of 0.5 per cent for sterilization and preservation. A saturated solution (approximately 5 per cent) in water may be employed, adding 1.0 cc. for each 10 cc. of vaccine to give the final concentration of 0.5 per cent.

3. Proceed as per the following example: 20 cc. of vaccine containing 1,000,000,000 per cc. is desired. The suspension contains 2,700,000,000 per cc.

$$\frac{1000 \times 20}{2700} = 7.0 \text{ cc. of suspension to be used}$$

4. In a sterile vial or bottle containing a few glass beads place: 7 cc. of suspension; 2 cc. of 5 per cent tricresol; 11 cc. of sterile saline.

5. If the vaccine is to be a mixed one, prepare separate vaccines of each organism in this manner and then mix equal parts of each.

6. Stopper with a sterile rubber cap, mix and place in the incubator at 37° C. for 24 hours.

7. Remove 0.5 cc. with a sterile syringe and needle and place it in a flask of at least 50 cc. of a suitable broth medium for sterility test. Incubate 24 to 48 hours and dispense the vaccine as ready for administration if sterile.

8. If not sterile, reculture the vaccine. As a general rule, 24 hours at 37° C. are sufficient unless spores are present.

**Sterilization by Heat.**—1. Stopper the vial or bottle with a rubber cap and immerse in a bath of cold water reaching above the level of the vaccine.

2. Place a thermometer in the bath, raise the temperature up to 60° C. and maintain it for 1 hour.

3. Make a culture of the vaccine for sterility as described above except that a tube of broth may be used.

4. If not sterile, the vaccine may be reheated for another hour although this may reduce its antigenic activity. As a general rule, 1 hour at 60° C. is sufficient unless contaminating spores are present, in which case it should be discarded.

### METHODS FOR DISPENSING VACCINES

1. It is quite convenient to dispense vaccines in vials or small bottles, stoppered with rubber caps, and labeled with name of organisms and number per cc.

2. The first dose may be of 0.1 cc. and subsequent doses gradually increased, as by 0.1 or 0.2 cc., according to reactions. These amounts are readily removed with a suitable syringe and needle after disinfecting the rubber cap with tincture of iodine or some other suitable disinfectant.

3. Some physicians prefer having vaccines dispensed in ampules.

4. The designated doses, as 0.1, 0.2, 0.3, 0.4 cc., etc., are placed in small sterile ampules with a sterile pipet and the volume in each brought up to 1 cc. by adding tricresolized saline solution (0.3 cc. of tricresol in 100 cc. of saline). The neck of each ampule is then sealed in a flame and each labeled with a number or the dose.

### THE KOLMER METHOD FOR THE PREPARATION OF AUTOGENOUS VACCINES

1. This method is based upon employing any exogenous toxins produced by bacteria as well as the organism themselves.

2. A pure culture of the organism is cultivated at 37° C. for at least 4 to 5 days in a hormone broth medium (do not use serum or blood broth). If there are 2 or more organisms, each is grown separately in pure culture and made up into separate vaccines, which are finally mixed together in equal proportions to give a single vaccine of desired numerical strength.

3. The broth culture is counted by the counting chamber or Wright method described above or estimated by a nephelometer prepared with broth.

4. Proceed as per following example: 20 cc. of vaccine containing 1,000,000,000 per cc. are desired. Count or estimate shows 5,200,000,000 per cc.

$$\frac{1000 \times 20}{5200} = 3.6 \text{ cc. of suspension to use}$$

5. In a sterile vial or bottle containing a few glass beads place: 3.6 cc. of broth suspension; 2.0 cc. of 5 per cent tricresol; 14.4 cc. of sterile saline.
6. If the vaccine is to be a mixed one prepare separate vaccines of each organism in this manner and then mix equal parts of each.
7. Stopper with a sterile rubber cap, mix and incubate at 37° C. for 24 hours. Do not heat at 55 to 60° C. as this destroys thermolabile toxins and particularly those produced by staphylococci.
8. Culture 0.2 cc. in at least 50 cc. of a suitable broth medium for sterility.
9. If sterile, the vaccine is ready for administration.
10. Vaccines prepared by this method are of a light brown color and may give slightly more local reaction at the site of infection. The first dose may be 0.1 or 0.2 cc. and subsequent doses slightly increased.

### PREPARATION OF TYPHOID-PARATYPHOID VACCINE

1. Use the Panama carrier strain of *B. typhosus* (National Institute of Health No. 58); the Kessel strain of *B. paratyphosus A* and the Rowland strain of *B. paratyphosus B*. These may be obtained from the Army Medical School, Washington, D. C.
2. Culture each strain in a tube of broth and examine for purity.
3. Inoculate Blake bottles of agar with each strain if a large amount of vaccine is to be prepared; for smaller amounts inoculate 24 slants of agar with *B. typhosus*, 24 with *B. paratyphosus A* and 24 with *B. paratyphosus B*.
4. Incubate for 2 to 3 days and examine for purity.
5. Prepare separate *heavy* suspensions of the 3 organisms by washing off the agar cultures with appropriate amounts of sterile saline solution.
6. Shake each suspension with sterile glass beads to break up clumps and filter each through sterile paper.
7. Count each suspension by the counting chamber method described above.
8. The finished vaccine should contain in each cc.:

1,000,000,000 *B. typhosus*  
 500,000,000 *B. paratyphosus A*  
 500,000,000 *B. paratyphosus B*

9. Proceed as per the following example: It is desired to make 500 cc. of finished triple vaccine. Count of typhoid suspension is 8,200,000,000 per cc. Count of para A suspension is 6,500,000,000 per cc. Count of para B suspension is 7,900,000,000 per cc.

$$\frac{1000 \times 500}{8200} = 61 \text{ cc. of typhoid suspension to be used}$$

$$\frac{500 \times 500}{6500} = 38.5 \text{ cc. of para A suspension to be used}$$

$$\frac{500 \times 500}{7900} = 31.6 \text{ cc. of para B suspension to be used}$$

10. In a sterile bottle place: 61 cc. of typhoid suspension; 38.5 cc. of para A suspension; 31.6 cc. of para B suspension; 75 cc. of 2 per cent solution of tricresol; 293.9 cc. of sterile saline solution.

11. Mix well. This gives 500 cc. of triple vaccine of the desired strength of each organism preserved with 0.3 per cent tricresol.

12. Place the bottle in a bath of cold water reaching above the level of the vaccine.

13. Place a thermometer and heat at 53° C. for 1 hour.

14. Culture 1 cc. in a small flask of broth for sterility; incubate for 48 hours. Make anaerobic culture and inoculate a rabbit and mouse for additional tests for sterility.

15. For adults the doses at weekly intervals are as follows by subcutaneous injection: 0.5, 1.0 and 1.0 cc.

### ISOLATION AND PREPARATION OF BACTERIOPHAGE

**Filters.**—1. The Berkefeld 3W and 5W are recommended, the 5W filter candles for small volumes and the 3W for larger amounts. The test tube containing the filtrate may be replaced by an empty sterile tube and the filter used 2 or 3 times for succeeding generations if the material is not too cloudy.

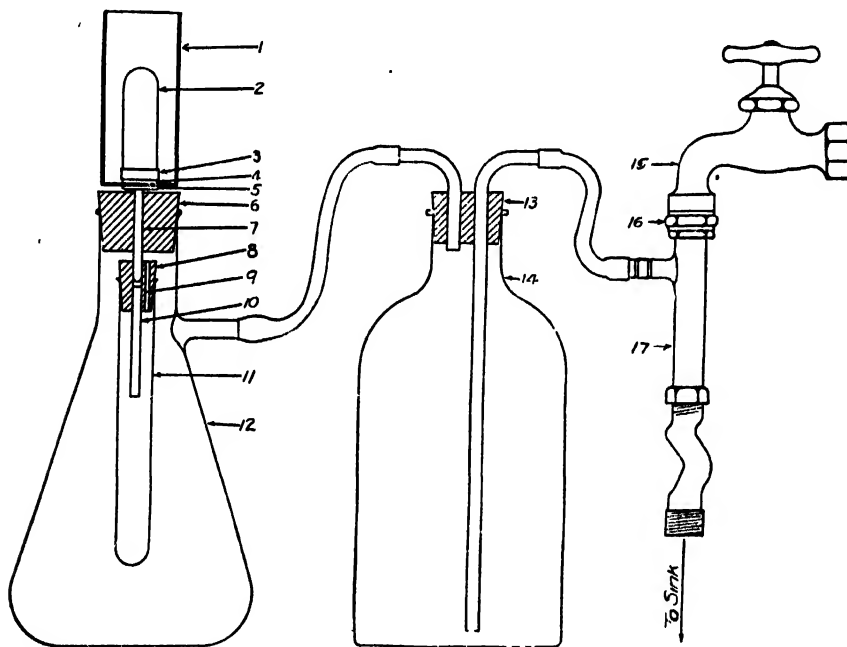


FIG. 209.—METHOD OF FILTRATION FOR THE PREPARATION OF BACTERIOPHAGE

(1) mantle; (2, 3, 4, 5) filter; (6) perforated rubber stopper; (7) neck of filter; (8) perforated rubber stopper in test tube (11); (12) filter flask with side-arm; (13) perforated rubber stopper in air-exhaustion bottle (14); (15) faucet; (16) connection for suction pump (17).

2. When the new filters are received the chalky deposit is removed from the outside by scrubbing gently under running water with a fine brush (nail brush) which has not been used with soap. Then the filters are boiled 15 to 20 minutes in distilled water 3 times. This tends to neutralize the filters and to cleanse them. The mantles are washed with soap and water and allowed to drain until they are dry.

3. They are assembled according to Figure 209. For ordinary set-ups a regular test tube holding 15 to 20 cc. is used but in preparing a large volume a larger test tube holding about 60 cc. is used. The air is drawn out of the Erlenmeyer flask by the use of suction and then out of the test tube through the second hole and between the 2 corks producing sufficient suction for filtration. The airway is so narrow and well protected that the filters when assembled and sterilized may be kept without being wrapped for several weeks before being used.

4. After being assembled the filters are sterilized in a steam autoclave at 121° C. for 1 hour.

5. After use the filters are dissembled and the 3 parts, the candles, mantles, and corks are boiled separately in tap-water for 20 minutes to disinfect them. If necessary they are autoclaved before being dissembled. After the boiling the filters are scrubbed and boiled as when new, the mantles are washed with soap and water and allowed to dry and the corks are rinsed. Then they are again ready to be assembled.

6. About every fifth time they are used before the 3 boilings in distilled water the candles are attached to the suction pump and distilled water is run through them for a few minutes to cleanse the interior.

**Method for Isolation of Bacteriophage from Feces.**—1. Disintegrate thoroughly by suspending about 5 grams in 50 cc. of broth (pH 7.4 to 7.8) and incubating 12 to 24 hours at 37° C.

2. Then centrifuge to remove large particles and filter through infusorial earth: provide a funnel with a folded filter paper large enough to receive at one time the entire volume to be filtered. Fill the filter with water to which has been added a small amount of infusorial earth. When the water has passed through, the paper is left coated with a thin layer of the infusorial earth, thus rendering the paper less permeable.

3. Then pass the filtrate through a sterile Berkefeld or similar filter. This removes the bacteria and the filtrate should be tested for bacteriophage for *B. coli*, *B. dysenteriae*, *B. typhosus*, staphylococci, streptococci, etc., as detailed in the descriptions given below.

**Method for Isolation of Bacteriophage from Sludge and Sewage.**—1. These are the most common sources and a bacteriophage for the colon bacillus can be almost invariably obtained.

2. Filter through paper or fine gauze to remove large particles.

3. Pass filtrate through a sterile Berkefeld filter. Culture 1 cc. in broth for 48 hours for sterility.

4. Or the following method may be employed: Inoculate a flask of broth with 10 cc. of sewage. Incubate overnight. Pass through an infusorial earth filter and then through a sterile Berkefeld to remove bacteria. Inoculate tubes carrying 20 cc. of double strength broth (pH 7.4 to 7.8) with 5 or 10 cc. of filtrate. Incubate 24 hours and filter through a sterile Berkefeld filter. The filtrate is then tested for bacteriophage.

**Method for Testing Feces and Sewage Filtrates for Bacteriophage.**—1. Prepare a suspension of the test organism from 18 to 24 hour agar slant culture of such density that 0.1 cc. added to 10 cc. of broth will give a faint perceptible cloud.

2. To each of 4 tubes carrying 10 cc. of broth (pH 7.6-7.8) add 0.1 cc. of the suspension of test organism. To 3 of the tubes add 0.5, 1, and 2 cc. of the filtrate to be tested for bacteriophage. The fourth tube is a culture control. To a fifth tube of 10 cc. of broth add 0.5 cc. of filtrate as a control on its sterility.

3. Incubate at 37° C. until there is a perceptible growth in the control (usually 4 hours). Observe carefully for clearing (lysis): ++++ = complete clearing; +++ = slightly cloudy; no sediment; ++ = perceptible clearing; some sediment; + = slightly clearer than the control.

4. Filter the first 2 tubes through sterile Berkefelds. Continue incubation of the third tube overnight. A reading is then made using the same scale. If lysis is not ++++, the process is repeated, using the filtrate obtained after the 4-hour incubation. When lysis is complete, a volume of phage is prepared, using the same materials and amounts, but setting up a large number of tubes.

**Method of Isolating Staphylococcus Bacteriophage from Pus.**—1. Place 1 cc. of pus in 20 cc. of broth or 5 cc. in a flask of broth (pH 7.6-7.8) and incubate at 37° C. overnight.

2. Filter through sterile sand, then sterile paper and finally a sterile Berkefeld filter (pus filtrate).

3. Prepare a thin suspension of the staphylococcus in broth.

4. In each of 5 sterile test tubes place 10 cc. of broth (pH 7.6-7.8).

5. Add 0.1 cc. of the bacterial suspension to the first 4 tubes.

6. To the first 3 add 0.5, 1 and 2 cc. of the pus filtrate respectively. Tube No. 4 is a culture control. To No. 5 add 0.5 cc. of pus filtrate as a control.

7. Incubate 4 to 24 hours and examine frequently. If bacteriophage is present lysis will occur in some or all of the first 3 tubes. No. 4 should show a good growth. No. 5 should be sterile and remain clear.

**Method for Testing and Dispensing Bacteriophage.**—1. Bacteriophage should not be used in treatment unless first shown to be lytic for the organism producing the infection. Commercial and stock phages should always be tested for lysis for the infecting organism before used.

2. Cultivate the organism in plain or blood agar for 18 to 24 hours and prepare a suspension in broth. If the growth is scant, use broth.

3. Place 9.5 cc. of broth or asparagin medium in each of 2 test tubes. To both add 0.1 cc. of the culture suspension sufficient to give a barely perceptible turbidity. To tube No. 1 add 0.5 cc. of the bacteriophage being tested.

4. Incubate both tubes at 37° C.; with staphylococcus or *B. coli* for 4 hours; with streptococcus about 6 to 12 hours.

5. Examine the first tube for lysis. The second or culture control tube should show a growth with increased turbidity.

6. If an asparagin bacteriophage is desired for intravenous use, it is advisable to carry it for two generations in asparagin medium, thus reducing the broth concentration to a negligible minimum.

7. Bacteriophage should be cultured by placing 1 cc. in broth and incubating 48 hours. None should be used which shows the slightest growth.

8. Very careful aseptic precautions should be exercised in dispensing. A preservative is not advisable as it may inhibit bacteriophage.

9. A bacteriophage made up with an organism from a patient's culture is called "autogenous." Stock bacteriophage is recommended in acute cases until the specific is prepared.

10. Bacteriophages should be kept in a refrigerator where they maintain potency for 4 months to 3 years.

**Stock Bacteriophages.**—1. These should be polyvalent and of high titer.

2. Stock staphylococcus and *B. coli* bacteriophages are most widely employed.

3. Streptococcus bacteriophage is highly specific and ++++ lysis is seldom obtained. Bacteriophage for hemolytic streptococci are prepared much more readily than for nonhemolytic types including *Streptococcus viridans*. Partially potent phages are sometimes used and have been found of some value in treatment.

4. In mixed infections, the bacteriophages are mixed in proportions indicated by the relative numbers of organisms in cultures.

**D'Herelle's Method for Titrating the Potency of Bacteriophage.**—1. To 60 cc. of broth add 0.2 cc. of an 18- to 24-hour broth culture of organism (bacterial suspension).

2. Set up 12 sterile test tubes and place 4.5 cc. of the bacterial suspension in each.

3. To No. 1 add 0.5 of bacteriophage.

4. Mix well and transfer 0.5 cc. to No. 2; mix well and transfer 0.5 cc. to No. 3 and so on to No. 12 from which discard 0.5 cc. Use a fresh sterile pipet for each transfer.

5. Include a control of 5 cc. with bacterial suspension.

6. Incubate 24 to 48 hours and examine for lysis.

**Method for Preparing B. Coli and Staphylococcus Bacteriophage for Therapy (New York Post-Graduate Hospital).**—1. The medium is plain beef infusion broth adjusted to pH 7.4 to 7.8. If for subcutaneous or intravenous administration and in case of protein allergy, an asparagin medium is preferred prepared as follows:

Asparagin (Merck) .....	3 gms.
Magnesium sulphate .....	2 gms.
Sodium chloride .....	4.5 gms.
Bipotassium hydrogen phosphate.....	2 gms.
Distilled water (neutral).....	1000 cc.

Dissolve in the water; bring to a boil; adjust to pH 7.6; autoclave at 121° C. for 15 minutes; filter through double paper; tube; autoclave a second time at 15 pounds for 30 minutes (final pH 7.0-7.2); should be clear.

2. Two pure cultures of each organism isolated from the case should be prepared on agar slants. One culture is to be kept in ice-box, while phage is being prepared, in case the other becomes contaminated.

3. Use 18- to 24-hour old agar slant cultures. Cover slant with broth making bacterial suspension. If the organism is *B. coli* set up 3 tubes of broth:

4. To all 3 tubes add 0.1 cc. of a light suspension of the case organism, enough to produce just a perceptible cloudiness.

5. To each of 2 tubes then add 0.5 cc. of mixed coli phage and label first generation. The third tube receives no phage but serves as a control. Shake and incubate at from 32° C. to 37° C. After 4 to 5 hours there should be a good growth in control tube. Take readings of phage tubes by comparing cloudiness with control:

0—No lysis—tube with phage not clearer than control.

±—Possible lysis—tube with phage possibly clearer than control.

1+—Little lysis—tube with phage definitely clearer than control.

2+—Semi lysis—tube with phage halfway between clear and control.....

- 3+—Good lysis—tube with phage  $\frac{3}{4}$  between clear and control.
- 4+—Complete lysis—tube with phage crystal clear.
- 1+ and 2+—May have sediment at bottom.
- 3+ and 4+—Have no sediment at bottom.

6. Whether there is lysis or not, filter one tube at this time. Put the other tube back in incubator for later readings. Filtrates should be tested for sterility.

7. The following day set up second generation in the same way, using 0.5 cc. of first generation filtrate instead of phage mixture, using fresh 24-hour old culture of organism made the day before. By successive generations and filtrations, the potency of a phage may be increased. The aim is to obtain 4+ (clear) lysis for 24 hours' incubation or longer. This is not always possible but frequently is accomplished.

8. Staphylococcus bacteriophage is prepared in the same manner except that 0.5 cc. staphylococcus phage mixture is used instead of coli phage. Complete lysis is obtained in the majority of cases from stock phage in the first generation with this organism. If organism is resistant, it is not usually possible to prepare a phage by successive filtrations.

9. If the organism is susceptible a volume of phage for therapy may be obtained by setting up 6 or more tubes and a control in the usual way and filtering into a larger sized tube, or larger amounts may be prepared by using 250 cc. of broth with corresponding amounts of culture and stock phage.

**Method for Preparing Streptococcus Bacteriophage for Therapy (New York Post-Graduate Hospital).**—1. The preparation of streptococcus bacteriophage presents much more difficulty.

2. The transplant of the organism is generally carried along in plain broth. A 24-hour growth is employed in the test for susceptibility to bacteriophage.

3. Five sterile test tubes, 3 of which contain approximately 10 cc. of plain broth are employed. If the phage is for subcutaneous or intravenous administration, and in case of protein allergy, the asparagin medium previously described is preferred. To each of the 2 empty tubes 10 cc. of a mixed streptococcus bacteriophage are added. To the third, fourth and fifth tubes, each of which contains broth, are added respectively 2 cc., 1 cc., and 0.5 cc. of the bacteriophage.

4. To each of these 5 tubes and to 1 tube containing only broth is added 0.1 cc. of the 24-hour growth of the streptococcus to be tested.

5. All tubes are shaken well and incubated for 18 hours at 32 C. to 37° C. A reading is taken at this time using the tube with no bacteriophage in it as a control.

6. Then the 5 tubes containing bacteriophage are filtered through a Berkefeld filter pouring off the top and leaving the sediment at the bottom.

7. This filtrate is the filtrate of the first generation and is used to set up the second generation in the same way, using the filtrate in place of the phage. Ordinarily one finds a partial lysis in the whole filtrate in the second generation. It is usually possible to enhance the bacteriophage by these serial filtrations so that it will produce a 4+ lysis in the whole filtrate and a partial lysis in the tubes with small amounts of filtrate.

8. Occasionally after serial filtration the phage will adapt itself so that a small amount will give complete lysis. Then it is possible to prepare large quantities as in working with the *B. coli* and staphylococcus. Ordinarily, however, it is necessary to repeat the set-ups and filter gaining only small volumes at a time.



## METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF MILK

**Principles.**—1. The methods here given are the standard methods of milk analysis of the American Public Health Association and the Association of Official Agricultural Chemists. They are given herewith for guiding the examination of milk in clinical laboratories, especially those connected with hospitals.

2. The total bacterial count continues to be of most value in the bacteriological examination of milk and is especially useful as a measure of the care with which milk is collected and kept until used.

3. Methods for the detection of tubercle bacilli in milk are described on page 463. Unfortunately, however, there are no thoroughly reliable methods at present for the detection of typhoid and dysentery bacilli, *Br. abortus*, and other pathogenic organisms known to be sometimes transmitted by milk. It is still necessary to rely mainly for the elimination of these upon thorough pasteurization, veterinary inspection of herds, and the medical examination and supervision of dairy employees with special reference to typhoid carriers.

4. It is true, however, that the presence of mastitis in cows is sometimes to be detected by finding exceedingly large numbers of long-chained streptococci and pus cells when due care is taken to examine the milk within 6 hours after collection or when carefully refrigerated to prevent multiplication. Due care, however, must be exercised in the examination of sediments secured by centrifuging against mistaking normal streptococci and those contained in butter starters or derived from dirty milking machine tubes, for pathogenic streptococci.

### STANDARD PLATE METHOD FOR TOTAL BACTERIAL COUNTS

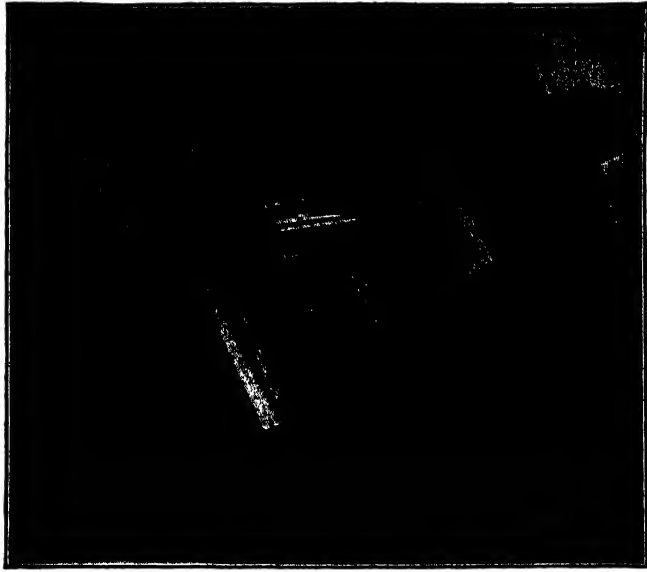
1. If bottled milk is to be examined, the bottle should be immediately iced. In case the milk is in bulk, a sample should be taken after thorough mixing. A sterile glass tube long enough to reach from the top to the bottom of the container to be sampled is very satisfactory. The tube is lowered to the bottom and the end closed with the thumb or a finger to hold the contents in the tube, which is then placed in a sample bottle. The sample bottle should be sterile and large enough to hold the entire amount in the tube. Sample bottles should be glass stoppered as cotton plugs are not satisfactory. *Do not collect less than 10 cc.*

2. If the sample is not to be examined immediately, place it in cracked ice so as to cool promptly to near the freezing point. Prepare dilution bottles to contain 99 cc. of water after sterilization in the autoclave. They should have rubber or glass stoppers. The number of bottles will depend upon the number of samples to be examined and dilutions desired.

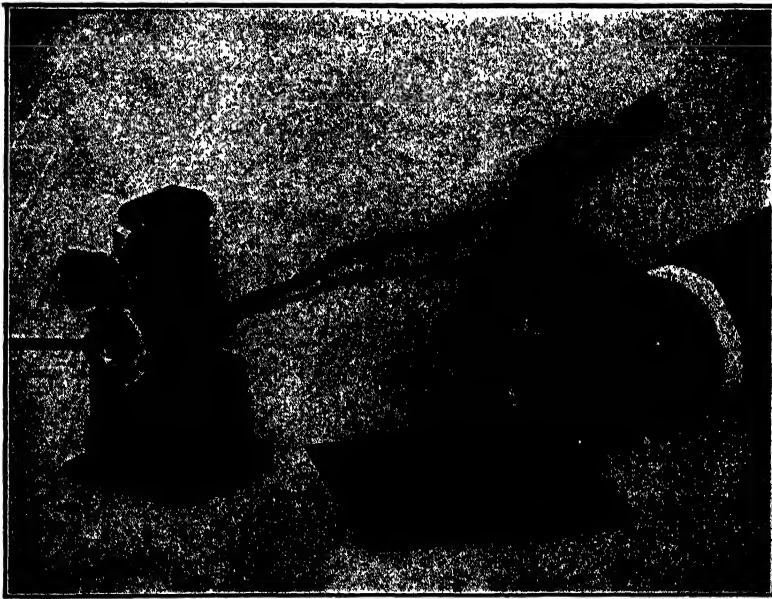
3. Shake sample 25 times, each shake being an up and down excursion of about 1 foot. Then immediately transfer 1 cc. to dilution bottle No. 1 (this makes a dilution of 1:100).

4. Shake dilution No. 1 25 times and transfer 1 cc. to dilution bottle No. 2. At the same time transfer 1 cc. and 0.1 cc. to 2 empty sterile Petri dishes. Mark the plates 1:100 and 1:1000.

5. Shake dilution No. 2 and transfer 1 cc. and 0.1 cc. to 2 empty Petri dishes and mark them 1:10,000 and 1:100,000. A special pipet is recommended which delivers 1.1 cc.



**FIG. 210.—TRENNER COLONY COUNTER AND SELECTOR**



**FIG. 211.—ROBINSON'S COLONY COUNTER**

6. Melt nutrient beef extract agar with a pH of 6.6 and cool to between 40° and 45° C.

7. Pour 10 cc. into each plate and mix with the diluted milk by gently rotating. Sufficient agar should be used to avoid drying out. An excess will favor the spreading of surface colonies.

8. Incubate all plates for 48 hours at 37° C. in an inverted position.

9. Select plates showing between 30 and 300 colonies. Count the number of colonies on the plates, using a lens magnifying about 2.5 diameters. A colony counting chamber with uniform illumination and with standard ruling should be used (Fig. 210). If the number of colonies exceeds 300, a fraction of the plate can be counted and the number multiplied by the factor and then by the dilution. Plates with less than 20 colonies should not be counted unless no others are available. When many samples are counted daily, Robinson's electric counting device may be found useful and time-saving (Fig. 211). In case of doubt, the compound microscope may be used to distinguish between colonies and debris.

10. Multiply the numbers of colonies by the dilution marked on the plate. This will give the number of bacteria per cc. of milk. Use only 2 significant left-hand digits in any report. Raise to the next highest round number but never lower.

11. *A series of at least 4 or more samples should be examined before judging the quality of a given milk supply.*

12. In addition to other requirements, the bacteria counts required before delivery of various grades of milk according to the standard milk ordinance of the United States Public Health Service, are as follows:

Certified Milk: Less than 10,000 per cc.

Grade "A" Raw: Not over 50,000 per cc.

Grade "B" Raw: Not over 200,000 per cc.

Grade "C" Raw: Not over 1,000,000 per cc.

Grade "D" Raw: Not over 5,000,000 per cc.

Grade "A" Pasteurized: Not only 10,000 per cc.

Grade "B" Pasteurized: Not over 100,000 per cc.

Grade "C" Pasteurized: Not over 500,000 per cc.

### DIRECT MICROSCOPIC COUNT OF BACTERIA

**Breed Method.**—1. The collection of sample is the same as described above for the plate count.

2. Thoroughly shake the sample and deposit 0.01 cc. of the milk on a clean slide by means of a special pipet (Fig. 212).

3. Spread the milk evenly over an area of 1 square centimeter with a clean stiff needle. The slide can be laid on paper ruled in 1 centimeter squares or on any ruled guide plate (Fig. 213). This ruling will show the area to be covered by the smear.

4. Dry the film in a warm place. Avoid excess heat as it may cause the film to crack. The drying should be complete within 5 to 10 minutes.

5. Dip the slide in xylol to remove the fat (at least 1 minute). Drain and allow to dry.

6. Place in 90 per cent alcohol for 1 or more minutes.
7. Transfer to Löffler's methylene blue for 5 minutes (to overstain).
8. Rinse in water and then decolorize with alcohol. Check the decolorization by observation to avoid overdecolorizing. When properly done the background should show a faint pale blue. If decolorization is carried too far, the smear can be restrained.



FIG. 212.—BREED AND BREW CAPILLARY  
PIPET

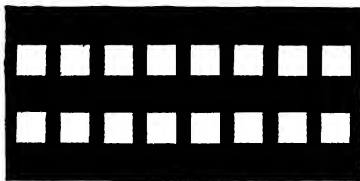


FIG. 213.—BREED AND BREW GUIDE  
PLATE

9. Or the following stain, devised by Newman, may be employed:

Methylene blue (certified powder).....	1.12 gm.
Ethyl alcohol (95 per cent).....	54.00 cc.
Tetrachlorethane (tech.) <sup>1</sup> .....	40.00 cc.
Glacial acetic acid .....	6.00 cc.

Add the alcohol to the tetrachlorethane in a flask and bring to a temperature not to exceed 70° C. (If it is desired to use methyl alcohol the temperature should not be raised to more than 55° to 60° C.) Add the warm mixture to the powdered methylene blue. Shake vigorously until the dye is completely dissolved; then add slowly the glacial acetic acid to the cold solution. Agitate the flask during addition of acid. Filter the entire volume through a 15 centimeter filter paper. Keep in tightly stoppered bottle. Allow the stain to act for 30 seconds. Steps 5 and 6 of the above may be omitted as the fat is removed by the stain.

10. Adjust the microscope so the field of vision is 0.205 millimeter in diameter. This can be done by using a stage micrometer with a 1.9 millimeter (oil-immersion) objective and a 6.4 × ocular; adjust the tube until the field has the required size.

Count the number of bacteria seen in 30 fields. Each field represents one three hundredth thousandth part of a cc. of the milk.

$$\frac{\text{number of bacteria}}{30} \times 300,000 = \text{number of bacteria per cc.}$$

or

$$\text{number of bacteria} \times 10,000$$

11. For some purposes, especially when examining low count milk, it is advisable to use a special ocular micrometer with circular ruling divided into quadrants (Fig. 214). Adjust the microscope so the diameter of the circle is 0.146 millimeter. Count 60 fields and multiply the number of bacteria by 10,000.

12. This direct microscopic method is excellent for making a rapid survey of either raw or pasteurized milk and should be more frequently used as a check on counts obtained by the plate method.

<sup>1</sup> Obtained from Eastman Kodak Co.

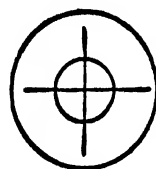


FIG. 214.—OCULAR  
MICROMETER DISK  
OF BREED AND  
BREW

**METHYLENE BLUE REDUCTION METHOD**

This test is also known as the reductase test. It is based on the principle that the color imparted to milk by a small quantity of methylene blue will disappear more or less quickly. The rate of this decolorization depends largely upon the reducing activity of bacteria. This in turn has been empirically correlated with the number of bacteria when the test is conducted under the usual circumstances and if other factors such as the temperature of the milk, are kept constant during the test.

1. Collect samples with same care as for other types of bacteriological examinations.

2. Place 10 cc. of the milk in a sterile test tube.

3. Add 1 cc. of methylene blue solution made by adding 1 tablet to 200 cc. of water (standard methylene blue tablets are prepared by the National Aniline Company and can be obtained from the usual supply houses).

4. Mix the dye thoroughly. This can be done by blowing through the pipet used to add the dye. The milk should now have a robin's-egg blue color.

5. Place the tubes in a water bath at 37° C.

6. Observe frequently. The end-point is to be taken as the time when the blue color has disappeared and the milk has regained its normal color. In the majority of cases the color disappears uniformly throughout the entire mass of milk. With certain samples the color may persist at the surface, or again it may persist at the bottom of the tube. In case the color disappears in an uneven manner, the end-point can be taken as the time when the milk after mixing shows no evidence of a blue color.

7. The results are interpreted as follows:

Class 1. Good milk: not decolorized in 5½ hours; developing as a rule, less than 500,000 colonies per cc. on agar plates.

Class 2. Milk of fair quality: decolorized in less than 5½ hours but not less than 2 hours; developing as a rule, 500,000 to 4,000,000 colonies per cc. on agar plates.

Class 3. Unsatisfactory milk: decolorized in less than 2 hours, but not less than 20 minutes; developing as a rule, 4,000,000 to 20,000,000 colonies per cc. on agar plates.

Class 4. Very unsatisfactory milk: decolorized in 20 minutes or less; developing as a rule, over 20,000,000 colonies per cc. on agar plates.

**METHODS FOR THE RECOGNITION OF HEMOLYTIC STREPTOCOCCI**

The following methods are useful for the examination of samples from cows suspected of having caused an outbreak of septic sore throat. They may also be used for routine control in those cases where raw milk is carefully guarded against infection with hemolytic streptococci.

1. Beef infusion agar is melted, cooled to 50° C. and 0.5 cc. of defibrinated blood added for each 10 cc. of agar.

2. Add 1.0 cc. and 0.1 cc. of milk respectively to 2 Petri dishes, overpour with agar and mix thoroughly by gently rotating the plate.

3. Incubate 24 hours at 37° C. Use an uninoculated plate as a sterile control. Plates showing no growth are incubated for an additional 24 hours.

4. Three types of streptococci may be encountered. The first group, the lactic acid streptococci, are not generally active on blood agar. The second type is that of

bovine mastitis which appears as a viridans (green) or weakly hemolytic colony. The third group is the highly hemolytic and contains the human type, of which *Streptococcus epidemicus* is a member. This organism is usually associated with septic sore throat epidemics.

5. Pick 1 or 2 hemolytic colonies to (a) glucose serum broth and to (b) sodium hippurate broth. *Glucose serum broth* is prepared by adding 1.0 per cent glucose to beef infusion broth. Two drops of serum are added at the time of inoculation. *Sodium hippurate broth* is prepared by adding 1.0 per cent sodium hippurate to pork or beef infusion broth.

6. After 2 days at 37° C. the final pH of the glucose serum broth culture is determined colorimetrically.

7. At the same time the sodium hippurate culture is examined by adding 1 part of the test reagent (12 per cent ferric chloride in a 2.0 per cent HCl solution) to 4 parts of the culture. Mix and observe after 10 minutes. A precipitate (ferric benzoate) indicates that the hippuric acid has been hydrolyzed.

8. If the suspicious organism produces a final pH of from 6.0 to 5.0 and fails to hydrolyze the hippurate, a moist Indian ink preparation should be made from the serum broth. Should an encapsulated organism be found, it is probably *Strept. epidemicus*. This organism usually ferments salicin but not mannitol.

#### METHOD FOR THE DETECTION OF TYPHOID AND PARATYPHOID BACILLI

1. Leifson recommends culturing 9 parts of milk in 1 part of selenite enrichment medium prepared in a concentration 10 times stronger than usual. If the milk is of poor quality it is advised to dilute at 1:4 with sterile water and add 9 parts of this dilution to 1 part of the concentrated medium.

2. Incubate at 37° C. for 18 to 24 hours and then prepare surface streak plates of desoxycholate-citrate agar or bismuth sulphite agar. If suspicious colonies are found, apply further tests for typhoid and paratyphoid bacilli as described on pages 471 to 474.

# METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF WATER

**Principles.**—1. The methods here given are the standard methods for the examination of water adopted by the American Public Health and American Water Works Associations.

2. From the standpoint of the clinical laboratory, examinations for bacilli of the coli-aerogenes group of fecal origin are of most value in relation to the spread of typhoid fever, cholera, and dysentery.

3. Direct examinations for typhoid and paratyphoid bacilli have been previously referred to, but are of so very little value that most reliance is placed upon finding colon bacilli as indicative of possible contamination with human fecal material.

## COLLECTION

1. Samples for bacterial analysis shall be collected in bottles which have been cleansed with care, rinsed in clean water, and sterilized.

2. Great care must be exercised to have the samples representative of the water to be tested and to see that no contamination occurs at the time of filling the bottles or prior to examination.

3. Because of the rapid and often extensive changes which may take place in the bacterial flora of bottled samples when stored even at temperatures as low as 10° C., it is urged, as of importance, that all samples be examined as promptly as possible after collection.

4. The time allowed for storage or transportation of a bacterial sample between the filling of the sample bottle and the beginning of the analysis should be *not more than 6 hours for impure waters* and *not more than 12 hours for relatively pure waters*. During the period of storage, the temperature shall be kept between 6° C. and 10° C. Any deviation from the above limits shall be so stated in making reports.

## TOTAL BACTERIAL COUNTS

1. Have ready the following: (a) Nutrient extract agar or nutrient gelatin with pH of 6.4 liquefied and cooled to 42° C. (b) Dilution bottles containing 9 cc. or 99 cc. of water and sterilized at 120° C. for 15 minutes.

2. When dilutions are made the sample bottle should be shaken vigorously 25 times and 1 cc. withdrawn and added to the proper dilution bottle as required. Each dilution bottle shall be shaken vigorously 25 times before a second dilution is made from it or before a sample is removed for plating.

3. Plating should be done immediately after making dilutions. After vigorous shaking 25 times, 1 cc. of the sample or dilution shall be placed in the Petri dish. Ten cc. of liquefied medium (agar or gelatin) at a temperature of 42° C. shall be added to the Petri dish. The cover of the Petri dish shall be lifted just enough for the introduction of the pipet or culture medium, and the lips of all test tubes or flasks used for pouring the medium shall be flamed. The medium and sample in the Petri dish shall be thoroughly mixed and uniformly spread over the bottom of the Petri dish by tilting and rotating the dish. All plates shall be solidified as rapidly as possible after pouring and placed immediately in the incubator.

4. *Gelatin plates* shall be incubated for 48 hours at 20° C. in a dark, well-ventilated incubator in an atmosphere practically saturated with moisture.

Agar plates may be used for counts made either at 20° C. or 37° C. The time for incubation at 20° C. shall be 48 hours and at 37° C. 24 hours. The incubator shall be dark, well-ventilated and the atmosphere shall be practically saturated with moisture. Glass-covered plates shall be inverted in the incubator. Any deviation from the above described method shall be stated in making reports.

In making report of the water examination the medium used for the total count should be stated, *i.e.*, whether gelatin or agar, and the temperature of incubation given.

5. In preparing plates, such amounts of the water under examination shall be planted as will give from 30 to 300 colonies on a plate; and the aim should be to always have at least 2 plates giving colonies between these limits. Where it is possible to obtain plates showing colonies within these limits, only such plates should be considered in recording results, except where the same amount of water has been planted in 2 or more plates, of which one gives colonies within these limits, while the others give less than 30 or more than 300. In such case, the result recorded should be the average of all the plates planted with this amount of water. Ordinarily it is not desirable to plant more than 1 cc. of water in a plate; therefore, when the total number of colonies developing from 1 cc. is less than 30, it is obviously necessary to record the results as observed, disregarding the general rule given above.

Counting shall in all cases be done with a lens of 2.5 diameters' magnification, with a focal distance of 3½ inches. The Engraver's Lens No. 146 made by the Bausch and Lomb Optical Company fills the requirements and is a convenient lens for the purpose.

6. In order to avoid fictitious accuracy and yet to express the numerical results by a method consistent with the precision of the work, the numbers of colonies of bacteria per cc. shall be recorded as follows:

From 1 to 50 shall be recorded as found.

From 51 to 100 shall be recorded to the nearest 5.

From 101 to 250 shall be recorded to the nearest 10.

From 251 to 500 shall be recorded to the nearest 25.

From 501 to 1000 shall be recorded to the nearest 50.

From 1001 to 10,000 shall be recorded to the nearest 100.

From 10,001 to 50,000 shall be recorded to the nearest 500.

From 50,001 to 100,000 shall be recorded to the nearest 1000.

From 100,001 to 500,000 shall be recorded to the nearest 10,000.

From 500,001 to 1,000,000 shall be recorded to the nearest 50,000.

From 1,000,001 to 10,000,000 shall be recorded to the nearest 100,000.

This applies to the gelatin count at 20° C. and to the agar counts at 20° C. and 37° C.

#### **DETERMINATION OF THE PRESENCE OF MEMBERS OF THE COLI-AEROGENES GROUP**

The coli-aerogenes group is to be considered as including all gram-negative non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media.



The test described under this heading is really a combination of 3 tests. The first is called the "presumptive test" and is conducted in all cases. The second is called the "confirmed test" and is used to confirm the first test when doubtful. The third is the "completed test," used when the results of the second test are doubtful.

**Presumptive Test.**—1. Inoculate a series of fermentation tubes containing lactose broth (nutrient broth containing 0.5 per cent lactose) with the following amounts of the water to be tested: 10, 1, 0.1, and 0.01, cc.

2. The amount of media should always equal at least twice the amount of water inoculated. Any type of fermentation tube can be used. The Durham tube with inverted vial is recommended. When required to examine larger amounts than 10 cc., as many tubes as necessary shall be inoculated with 10 cc. each.

3. Incubate at 37° C. for 48 hours.

4. Examine each tube at the end of 24 and 48 hours.

5. The production within 24 hours of gas occupying more than 10 per cent of the inverted vial in the fermentation tube constitutes a *positive presumptive test*.

6. If no gas is formed in 24 hours, or if the gas formed is less than 10 per cent, the incubation shall be continued to 48 hours. The presence of gas in any amount in such a tube at 48 hours constitutes a *doubtful test*, which in all cases requires confirmation.

7. The absence of gas formation after 48 hours' incubation constitutes a *negative test*.

**Confirmed Test.**—1. Streak or spread Endo or eosin-methylene blue plates from the tube which shows gas formation from the smallest amount of water tested. The transfer should be made as soon as possible after gas formation occurs. If gas formation occurs at the end of 24 hours, make transfer at that time. If at the end of 48 hours gas has formed in tubes containing less of the sample of water than at 24 hours, transfers should be made from these tubes.

2. Incubate at 37° C. for 18 to 24 hours.

3. The results are interpreted as follows: (a) If typical colonies have developed upon the plate within this period, the confirmed test may be considered positive. (b) If, however, no typical colonies have developed within 24 hours, the test cannot yet be considered definitely negative, since it not infrequently happens that members of the coli-aerogenes group fail to form typical colonies on Endo or eosin-methylene blue plates, or that the colonies develop slowly. In such case, it is always necessary to complete the test as described below.

**Completed Test.**—1. (a) *From typical plates:* From the Endo or eosin-methylene blue plates showing typical colonies, fish at least 2 colonies, transferring each to an agar slant and a lactose fermentation tube.

(b) *From atypical plates:* If no typical colonies appear upon the plate within 24 hours, the plate should be incubated another 24 hours, after which at least 2 of the colonies considered most likely to be organisms of the coli-aerogenes group whether typical or not shall be transferred to agar slants and lactose fermentation tubes.

2. Incubate the lactose broth fermentation tubes until gas formation is noted, the incubation not to exceed 48 hours. The agar slants shall be incubated 37° C. for 24 hours, when a microscopic examination shall be made of at least 1 culture, selecting ~~he~~ one which corresponds to one of the lactose broth fermentation tubes which has shown gas formation.

3. The formation of gas in lactose broth and the demonstration of gram-negative non-spore-forming bacilli in the agar culture shall be considered a satisfactorily completed test, demonstrating the presence of a member of the coli-aerogenous group. The absence of gas formation in lactose broth or failure to demonstrate gram-negative non-spore-forming bacilli in a gas-forming culture constitutes a negative test.

**Interpretation of the Results.—Presumptive Test.**—1. When definitely positive, that is, showing more than 10 per cent of gas in 24 hours, this test is sufficient:

(a) As applied to all except the smallest gas-forming portion of each sample in all examinations.

(b) As applied to the smallest gas-forming portion in the examination of sewage or of water showing relatively high pollution, such that its fitness for use as drinking water does not come into consideration. This applies to the routine examination of raw water in connection with control of the operation of purification of plants.

2. When definitely negative, that is, showing no gas in 48 hours, this test is final and therefore sufficient in all cases.

3. When doubtful, that is, showing gas less than 10 per cent (or none) in 24 hours, with gas either more or less than 10 per cent in 48 hours, this test must always be confirmed.

**Confirmed Test.**—1. When definitely positive, that is, showing typical plate colonies within 24 hours, this test is sufficient:

(a) When applied to confirm a doubtful presumptive test in cases where the latter, if definitely positive, would have been sufficient.

(b) In the routine examination of water supplies where a sufficient number of prior examinations have established a satisfactory index of the accuracy and significance of this test in terms of the completed test.

2. When doubtful, that is, showing colonies of doubtful or negative appearance in 24 hours, this test must always be completed.

**Completed Test.**—The completed test is required as applied to the smallest gas-forming portion of each sample in all cases other than those noted as exceptions under the "presumptive" and the "confirmed" tests.

The completed test is required in *all* cases where the result of the partially confirmed test has been doubtful.

**Note.**—1. In reporting a single test, it is preferable merely to record results as observed, indicating the amounts tested and the result in each, rather than to attempt expression of the result in number of organisms per cc. In summarizing the results of a series of tests, however, it is desirable, for the sake of simplicity, to express the results in terms of the number of coli-aerogenes organisms per cc., or per 100 cc. The number per cc. is the reciprocal of the smallest portion (expressed in cc.) giving a positive result. For example, the result 1 cc. plus, 0.1 cc. plus, 0.01 cc. negative, would be recorded as 10 per cc. An exception should be made in the case where a negative result is obtained in an amount larger than the smallest portion giving a positive result; for example, in a result such as 10 cc. plus, 1 cc. minus, 0.1 cc. In such case, the result should be recorded as indicating a number of coli-aerogenes organisms per cc. equal to the reciprocal of the portion next larger than the smallest one giving a positive test, this being a more probable result.

Where tests are made in amounts larger than 1 cc. giving average results less than 1 per cc., it is more convenient to express results per 100 cc.

2. Recent work seems to indicate that the coli-aerogenes group as herein defined consists of organisms of both fecal and nonfecal origin. Methods for making this distinction are the methyl red, Voges-Proskauer, indol, and sodium citrate tests, but none have been as yet adopted as standard. This statement should not be construed as detracting from the value of the group test as above described for the routine examination of water supplies.

### DIFFERENTIATION OF THE COLI-AEROGENES GROUP INTO FECAL AND NONFECAL TYPES

The coli-aerogenes group contains organisms of both fecal and nonfecal origin. For a satisfactory differentiation the following tests are required:

1. *Indol*.
2. *Voges-Proskauer*.
3. *Methyl Red*: Inoculate a 5 cc. portion of methyl red Voges-Proskauer broth. Incubate 24 hours at 37° C. Add 5 drops of methyl red indicator solution. A distinct red color is positive; a yellow color is negative.
4. *Sodium citrate*: Inoculate Koser's citrate broth with a loopful of culture or with a needle. Incubate for 3 days at 37° C. *B. coli* fails to grow in this medium.

### REACTIONS OF THE COLI-AEROGENES GROUP

	Indol	Methyl Red	Voges-Proskauer	Citrate
Fecal .....	+	+	.	—
Nonfecal .....	--	--	+	+

Intermediate types are common and difficult to interpret.

## METHODS FOR TESTING DISINFECTANTS

A very large number of methods have been proposed for testing the bactericidal and bacteriostatic (antiseptic) properties of disinfectants and the method of testing has a tremendous influence upon the results. For this reason one laboratory may report a substance as possessing a high disinfectant value and another that it is practically inert. It is easily possible, therefore, to influence greatly the value placed upon a disinfectant by the method of testing. It is hoped that the methods here given will prove serviceable for the purposes of the clinical laboratory.

The standardization of disinfectants and antiseptics is based upon their disinfecting power in comparison with phenol. The ratio is expressed as the *phenol coefficient*, which is a figure expressing the ratio of the germicidal efficiency of a disinfectant compared with phenol tested under identical conditions. It is not based on a comparison of different time intervals, but on a comparison of different concentrations acting for specified periods of time at designated temperatures.

At present there are in general use three methods of determining the phenol coefficient: The U. S. Hygienic Laboratory Method (Reprint No. 675, U. S. Pub. Health, Reg., 1921, 36, 1559), the Rideal-Walker Method, and the Method of the Food and Drug Administration (F. D. A. Method) given below.

The latter designed by Shippen and Reddish (Circular No. 198, U. S. Dept. of Agriculture, December, 1931) is based upon the Hygienic Laboratory and Rideal-Walker Methods and is particularly valuable in the curtailment of labor, time and material as well as being adapted to the use of *Staphylococcus aureus* and other organisms as well as for *B. typhosus* (*E. typhi*).

### FOOD AND DRUG ADMINISTRATION METHOD \*

**Test Organism and Culture Medium.**—1. The test organism is a 22 to 26 hour culture of *Eberthella typhi* (Hopkins strain) incubated and grown in nutrient broth at 37° C. The broth contains the following ingredients: 5 gm. of Liebig's beef extract, 5 gm. of chemically pure sodium chloride, and 10 gm. of Armour's Peptone (for disinfectant testing) in 1,000 cc. of distilled water. The mixture is boiled for 20 minutes, made up to original weight (or volume) with distilled water, and adjusted with NaOH to pH 6.8 using the colorimetric method. It is then filtered through paper, tubed (10 cc. of each tube) and the tubes plugged with cotton and sterilized at 15 pounds pressure for 40 minutes.

2. The test culture is transferred daily in this medium for not more than 1 month. At the end of each month, a fresh transfer is made from the stock culture. The stock culture is carried on agar slants of the same composition as the broth medium plus 1½ per cent Bacto-Agar (Difco) adjusted to pH 7.2 to 7.4. This medium is also filtered, tubed, plugged with cotton, sterilized and slanted. The stock culture is transferred once a month, and the test organism is taken from the month old stock culture. When the test organism has not been transferred daily, it is advisable to make 4 or 5 consecutive daily transfers in broth before using it for testing purposes, to be reasonably sure of its conforming to the phenol resistance requirements. When only one transfer has been skipped, the following transfer from the 48-hour culture is usually satisfactory for use after 24 hours. Transfers are made with the platinum loop used

\* U. S. Dept. Agriculture, Circular No. 198, Dec., 1931.

in the test. Only cultures giving readings within the following limits are considered satisfactory:

TABLE 23

Phenol	5 Minutes	10 Minutes	15 Minutes
1-90 .....	+	+	0
1-100 .....	+	+	+
or			
1-90 .....	0	0	0
1-100 .....	+	+	0

TABLE 24

Phenol	5 Minutes	10 Minutes	15 Minutes
1-90 .....	+	0	0
1-100 .....	+	+	+

The reading in Table 24 is that most usually obtained and is the most convenient.

**Phenol.**—The phenol used must meet the requirements of the U.S.P. and in addition the congealing point must not be below 40° C. A 5 per cent solution may be used as a stock solution if kept in a relatively cool place in well stoppered amber-colored bottles protected from the light. This 5 per cent solution should be standardized with decinormal bromine or with sodium bromide and bromate solution.

**Apparatus.**—1. Besides a number of accurately graduated pipets, 100 cc. glass-stoppered graduates or volumetric flasks are almost essential for the making of correct dilutions. All pipets and graduates should be standardized. The test tubes for containing the dilutions should be large enough to permit transfers being made without touching the sides with the transfer needle. Lipped pyrex (to withstand constant flaming) test tubes 25 by 150 mm. serve very well as these seeding or medication tubes.

2. A water bath for holding the dilutions at the desired temperature must be provided. To maintain the temperature practically constant during the period of the test, the bath should be made so as to contain a relatively large volume per surface area, and should be insulated. The lid is made with well-spaced holes admitting the 25 mm. tube, but not the lip.

3. The most convenient form of subculture tubes (tubes containing medium for incubating the tested organisms, as well as for growing the test culture) are ordinary nonlipped bacteriological test tubes 20 by 150 mm.

4. The racks for holding the subculture tubes may be of any convenient style. Blocks of wood with a series of holes bored in them are quite satisfactory. Dimensions depend somewhat on the size of the incubator, but the holes should be well spaced to insure quick selection and easy manipulation during the test. It is an added convenience to have the holes large enough to admit the medication tubes while dilutions are being made.

5. The transfers are made with 4 mm. (inside diameter) single loop of number 23B and S. gage platinum wire 1½ to 3 inches long, set in a suitable holder such as an aluminum or glass rod approximately 0.5 cm. in diameter.

**Procedure.**—1. One per cent stock dilutions of the substance to be tested (or any other convenient dilution of the disinfectant, depending on the strength) are made up, usually in glass-stoppered cylinders or volumetric flasks from which the individual dilutions are then prepared. For rapid routine work the final dilutions may be made directly in the medication tubes. In this case all excess over 5 cc. must be removed. For more precise work, and when high dilutions are required or volatile substances are dealt with, it is preferable to make up all of the dilutions in volumetric flasks, and then transfer 5 cc. of the final dilution to the medication tubes. The accompanying tables taken from the Hygienic Laboratory Method show how varying dilutions may be prepared from stock 5 per cent (1:20) and 1 per cent (1:100) solutions of the disinfectant being tested.

2. These tubes containing 5 cc. of each dilution (including the phenol control) are placed in the water bath at 20° C. for 5 minutes until the temperature of the bath is reached. Even slight variations in temperature may affect the results. The dilutions should cover the range of the killing limits of the disinfectants within 5 and 15 minute periods, and should at the same time be spaced sufficiently close together to insure the desired accuracy.

3. Five-tenths of a cubic centimeter of the test culture is then added to each of the dilutions at the time interval corresponding to the interval at which the transfers are to be made. Thus by the time 10 tubes have been seeded at 30-second intervals, 4½ minutes will have elapsed and a 30-second interval intervenes before the transference to the subculture is commenced. The culture is added from a graduated pipet holding sufficient culture to seed all the tubes in any one set. The pipet may be loosely plugged with cotton at the mouth end before sterilizing, as a precautionary measure. Unfiltered culture is used, but it should be thoroughly shaken 15 minutes before use, and allowed to settle. The temperature of the culture should be practically that of the water bath before being added.

4. In inoculating the medication tubes they should be held in a slanting position, after removal from the bath, and the culture run in without the tip of the pipet touching the disinfectant. The tip may be allowed to rest against the side of the tube just above the surface of the liquid.

5. The tubes are agitated gently but thoroughly after the addition of the culture to insure even distribution of the bacteria.

6. Five minutes from the time of seeding the first medication tube, transfer 1 loopful of the mixture of culture and diluted disinfectant from the medication tube to the corresponding subculture tube. To facilitate transfer of uniform drops of the medication mixture, the loop is bent to form a slight angle with the stem and the medication tube is held at an angle of 60 degrees. In other words, as the loop is withdrawn, its plane should be parallel with the surface of the liquid. At the end of 30 seconds, a loopful is transferred from the second medication tube to the second subculture tube and the process continued for each successive dilution. Five minutes from the time of making the first transfer, a second set of transfers is begun for the 10-minute period, and finally repeated for the 15 minute period.

7. Before each transfer the loop is heated to red heat in the Bunsen flame and the mouth of every tube is flamed. Sterilization of the loop is effected immediately after making the previous transfer (before replugging the tubes) to allow time for sufficient cooling. Time does not permit flaming the tubes after making the transfer.

## METHODS FOR TESTING DISINFECTANTS

5 cc. of disinfectant      95 cc. of distilled water      Solution A

		Solution A, cc.	Distilled Water, cc.		Solution A, cc.	Distilled Water, cc.		Solution A, cc.	Distilled Water, cc.		Solution A, cc.	Distilled Water, cc.
1: 20	=	20	+	0	or	10	+	0	or	4	+	0
1: 25	=	20	+	5	or	10	+	2.5	or	4	+	1
1: 30	=	20	+	10	or	10	+	5	or	4	+	2
1: 35	=	20	+	15	or	10	+	7.5	or	4	+	3
1: 40	=	20	+	20	or	10	+	10	or	4	+	4
1: 45	=	20	+	25	or	10	+	12.5	or	4	+	5
1: 50	=	20	+	30	or	10	+	15	or	4	+	6
1: 55	=	20	+	35	or	10	+	17.5	or	4	+	7
1: 60	=	20	+	40	or	10	+	20	or	4	+	8
1: 65	=	20	+	45	or	10	+	22.5	or	4	+	9
1: 70	=	20	+	50	or	10	+	25	or	4	+	10
1: 80	=	20	+	60	or	10	+	30	or	4	+	12
1: 90	=	20	+	70	or	10	+	35	or	4	+	14
1: 100	=	20	+	80	or	10	+	40	or	4	+	16
1: 110	=	20	+	90	or	10	+	45	or	4	+	18
1: 120	=	20	+	100	or	10	+	50	or	4	+	20
1: 130	=	20	+	110	or	10	+	55	or	4	+	22
1: 140	=	20	+	120	or	10	+	60	or	4	+	24
1: 150	=	20	+	130	or	10	+	65	or	4	+	26
1: 160	=	20	+	140	or	10	+	70	or	4	+	28
1: 170	=	20	+	150	or	10	+	75	or	4	+	30
1: 180	=	20	+	160	or	10	+	80	or	4	+	32
1: 200	=	20	+	180	or	10	+	90	or	4	+	36
1: 225	=	20	+	205	or	4	+	41	or	2	+	20.5
1: 250	=	20	+	230	or	4	+	46	or	2	+	23
1: 275	=	20	+	255	or	4	+	51	or	2	+	25.5
1: 300	=	20	+	280	or	4	+	56	or	2	+	28
1: 325	=	20	+	305	or	4	+	61	or	2	+	30.5
1: 350	=	20	+	330	or	4	+	66	or	2	+	30
1: 375	=	20	+	355	or	4	+	71	or	2	+	33.5
1: 400	=	20	+	380	or	4	+	76	or	2	+	38
1: 450	=	20	+	430	or	4	+	86	or	2	+	43
1: 500	=	20	+	480	or	4	+	96	or	2	+	48

For this reason, care in transferring and seeding is necessary. Due caution is observed to prevent either the seeding pipet or the transfer needle from touching the sides or mouth of these.

8. After completion of the transferring, the subculture tubes are incubated at 37° C. for 48 hours and results read. Macroscopic examination suffices for this, but occasionally agglutination with antityphoid serum will aid in reading doubtful results. A 3-day incubation period or agar streak or microscopic examination may be resorted to in determining feeble growth, especially when organisms other than *Eberthella typhi* are used.

There are certain types of germicidal agents, such as many of the mercury compounds, which give very high results by phenol coefficient tests. Due to the high inhibitory value of such substances in preventing growth in the subcultures these figures are frequently misleading. For germicides used in the disinfection of such objects as surgical instruments, this is of particular importance and must be taken into account. Failure to appreciate this characteristic of certain compounds is much more likely to lead to error when *Staphylococcus aureus* is used rather than *Eberthella typhi* as the test organism. That false values may not be obtained for products of this type, or for any other disinfectant giving suspiciously high results, the subcultures should contain very large amounts of medium (not less than 200 cc.) or they should be transferred by





expressing the dilution) of the disinfectant capable of killing *Eberthella typhi* in 14 minutes but not in 5 minutes, by the greatest dilution of the phenol showing the same results, that is, by the phenol control. Thus, if the results were as follows:

	5 Minutes	10 Minutes	15 Minutes
<b>DISINFECTANT (X)</b>			
1-300 . . . . .	0	0	0
1-325 . . . . .	+	0	0
1-350 . . . . .	+	0	0
1-375 . . . . .	+	+	0
1-400 . . . . .	+	+	+
<b>PHENOL</b>			
1-90 . . . . .	+	0	0
1-100 . . . . .	+	+	+

The phenol coefficient would be  $\frac{350}{90}$  equals 3.89

2. If none of the dilutions shows growth in 5 minutes and killing in 10 minutes, the hypothetical dilution may be estimated in certain cases. This may be done only when any 3 consecutive dilutions show the following results: The first—no growth in 5 minutes; the second, growth in 10 minutes but not in 15 minutes, and the third, growth in 15 minutes. Example:

	5 Minutes	10 Minutes	15 Minutes
<b>DISINFECTANT (X)</b>			
1-300 . . . . .	0	0	0
1-350 . . . . .	+	+	0
1-400 . . . . .	+	+	+
<b>PHENOL</b>			
1-90 . . . . .	0	0	0
1-100 . . . . .	+	+	0

The estimated phenol coefficient would be  $\frac{325}{95}$  equals 3.42.

3. To avoid giving an impression of fictitious accuracy, the phenol coefficient is calculated to the nearest 0.1 unless the coefficient is less than 1.0. Thus, in the examples cited above, the phenol coefficients would be reported as 3.9 and 3.4 instead of 3.89 and 3.42.

In the preceding description, *Eberthella typhi* has been mentioned as the test organism. Wherever any expression of phenol coefficient occurs in literature, on labels, etc., it is assumed to mean the *E. typhi* phenol coefficient unless otherwise stated. It is, however, the distinct intention of the U. S. Dept. of Agriculture not to limit the test to the use of one organism. In fact, the test has been found adaptable to the use of a wide variety of bacterial species in the determination of phenol coefficients. In cases where some of the more strictly parasitic bacteria are used, modifications in media are necessitated, and, of course, a change in the phenol dilutions. Therefore,

discussion of the exact technic is here omitted, with the exception of that for *Staphylococcus aureus*. When any test organism other than *E. typhi* is used it should be distinctly designated when stating the phenol coefficient.

**Tests with *Staphylococcus Aureus*.**—1. *S. aureus* has been found to be an extremely useful organism for testing disinfectants and antiseptics and has been used for this purpose for a number of years. When substituted in the above test the technic remains exactly the same. The phenol dilutions, however, must be changed. The resistance of any strain of *S. aureus* used in this test must come within the following limits: At 20° C. it must survive a 1 : 60 dilution of phenol for 5 minutes and a 1 : 70 dilution for 15 minutes. The following is the minimal resistance that would be acceptable:

	5 Minutes	10 Minutes	15 Minutes
PHENOL			
1:60 . . . . .	+	0	0
1:70 . . . . .	+	+	+

2. In the bacteriological examination of disinfectants, the *Eberthella typhi* and the *S. aureus* phenol coefficients give, in general, sufficient information to render tests with other organisms unnecessary, except in special instances. The commonly accepted criterion that disinfectants for general use be employed at a dilution equivalent to the germicidal efficiency of 5 per cent phenol against *E. typhi* (that is, 20 times the *E. typhi* phenol coefficient) allows a reasonable margin of safety for the destruction of infective agents likely to be the object of general disinfection about premises with the possible exception of *Mycobacterium tuberculosis*. *S. aureus*, due to its ubiquity, resistance and ever ready tendency to cause infection, should always be employed in testing those substances recommended for personal use or as application for wounds. If the disinfectant is recommended for use externally the temperature of test should be 20° C. but where such substances are recommended for use in the body cavities such as for mouth washes, gargles, douches, etc., this test should be conducted at 37° C. In such case the test should be designated as "The F. D. A. method (special) *S. aureus* 37° C." At body temperature the *S. aureus* should show the following resistance to phenol:

	5 Minutes	10 Minutes	15 Minutes
1-80 . . . . .	+	0	0
1-90 . . . . .	+	+	+
or			
1-80 . . . . .	+	0	0
1-90 . . . . .	+	+	0

## KOLMER BACTERIOSTATIC METHODS

This test is of extreme simplicity and yields sharply defined results. It determines the highest dilution of a disinfectant capable of restraining the growth of the test organism for a stated period of time and is of particular value for comparing the antiseptic properties of various chemical agents.

**Method Employing Nutrient Bouillon.**—For tests with staphylococci, *B. anthracis*, *B. typhosus*, *B. coli* and such hardy organisms, plain beef extract broth with a pH of 7.1 may be employed; Kligler has used a medium prepared of 1 per cent Fairchild's peptone, 0.5 per cent dibasic potassium phosphate, 0.5 per cent sodium chloride and 0.1 per cent glucose with a constant pH of 7.1. The reaction of any medium employed is particularly important as the results may be greatly modified by this factor. For streptococci and pneumococci, hormone broth (Huntoon) with 0.1 per cent dextrose and a pH of 7.7 is to be preferred, and for tubercle bacilli, the ordinary 5 per cent glycerin-hormone broth employed in the manufacture of Koch's tuberculin should be used. *It is always advisable to determine beforehand that the organism will grow well in the medium employed before the tests are conducted.*

1. As a general rule, 10 dilutions are advisable and for this purpose 10 sterile test tubes are arranged for each compound, including a set for the bichloride of mercury or phenol controls.

2. In all tubes except No. 1 of each series, place 1 cc. of sterile distilled water.

3. In tubes 1 and 2 place 1 cc. of the stock solution of disinfectant which is ten times higher than the final dilutions desired. Mix No. 2 and transfer 1 cc. to No. 3 and so on to No. 10 from which discard 1 cc.

4. In a flask of 99 cc. of the culture medium, place 1 cc. of a 24 to 48 hour broth culture of the test organism; with such organisms as streptococci and pneumococci it is well, however, to seed by mixing 5 cc. of a broth culture with 95 cc. of the culture medium. Mix well and add 9 cc. to each tube of the set; the remaining 10 cc. are placed in a sterile tube as a control on the culture.

5. In tests employing tubercle bacilli, it is better to add 9 cc. of sterile medium to each tube and then to seed by floating a loopful of bacilli on the surface of each.

6. The final dilutions are now ten times higher in each tube; for example, 1 cc. of 1 : 1000 stock solution in No. 1 becomes 1 : 10,000 and the final dilution in No. 10 is 1 : 5,120,000.

7. The tubes are incubated and the results recorded daily for 5 days. When the medium remains clear the result is recorded as *minus*; when a visible growth appears, the result is recorded as *plus*. At the end of this period the tubes may be cultured by transferring several loopsful to slants of a solid medium to determine whether the organisms have been killed or merely restrained. In this manner a bactericidal test is conducted at the same time in which a few organisms have been exposed to the disinfectant for 5 days. The control should be subcultured at the same time to make sure that the organisms are viable.

8. The results are expressed according to the highest bacteriostatic and bactericidal dilutions and also according to the bichloride or phenol coefficients previously described.

9. After obtaining in this manner an approximate idea of the activity of the compound under study, a second series of dilutions is prepared in which the variations from tube to tube are less marked.

**Method Employing Serum, Blood, Ascites Fluid or Muscle Extract.**—These tests are conducted in exactly the same manner as described above except that to 89 cc. of a suitable broth medium are added 10 cc. of sterile serum, blood or ascites fluid; the mixture is then seeded with 1 cc. of a broth culture of the test organism;

with such organisms as the pneumococcus and streptococcus, however, it is generally advisable to use 85 cc. of both, 10 cc. of serum, blood or ascites fluid with 5 cc. of broth culture of the test organism. This gives a 10 per cent solution of serum, blood, or ascites fluid, and while more or less may be employed as desired, yet experience has indicated that the above is satisfactory for eliciting the influence of these substances upon the degree of antibacterial activity of disinfectants.

Numerous experiments have shown that while the bacteriostatic and bactericidal activity of various disinfectants is reduced to a greater extent on a 50 per cent than on a 5 per cent dilution of serum, defibrinated blood, or ascites fluid, yet for all practical purposes a 10 per cent solution is satisfactory for this purpose and in view of the large amounts required, is to be preferred from the standpoint of economy. With muscle extracts, however, it may be advisable to use equal parts with broth (50 per cent) but muscle extract may be prepared so cheaply and quickly, as required, that the questions of economy and supply are not involved. These preparations are likewise slightly cloudy, but not usually to a degree sufficient for interfering with the ease and accuracy of readings. A menstruum containing 10 per cent of a muscle extract prepared of ordinary beef or veal requires a far higher concentration of disinfectants than a menstruum of 10 per cent blood or serum; this is doubtless due to the presence of large numbers of various bacteria in addition to the test organism.

Other special media like hormone-dextrose broth with brain tissue, ascites broth with sterile kidney, etc., may be employed in tests of this kind, and while the results vary according to the constitution of the medium, yet if mercuric chloride or phenol are included in each and every test, the results may be expressed in terms of the coefficients or indices. The coefficients, however, will vary according to the chemical nature of the compound as this is influenced by the constitution of the medium; for example, some compounds of mercury, like mercurophen and metaphen, maintain a higher degree of bacteriostatic activity in a serum, blood, or brain medium than mercuric chloride and thereby yield higher coefficients. In other words, while the bactericidal activity of almost all disinfectants is reduced in the presence of serum, blood, muscle extract, etc., the degree of reduction varies considerably among different compounds.

**Method Employing Solid Media; Mycostatic Test.**—It is very easy to employ solid culture media in this technic for such organisms that grow better on solid than on fluid media, such as the various yeasts and molds.

In conducting this test, a series of dilutions of the disinfectant in amounts of 1 cc. in sterile distilled water are prepared in sterile test tubes as previously described. To each tube and a control are now added 9 cc. of an appropriate agar medium cooled to 42 to 45° C.; the contents are well mixed and allowed to harden in slants. The dilution in each tube is now 10 times higher; or 4 cc. of medium may be added to each tube, which renders the final dilution in each 5 times higher.

For such organisms as staphylococci, *B. typhosus*, *B. coli*, etc., plain 2 per cent agar (pH 7.1) may be employed; for streptococci and pneumococci a hormone-dextrose (0.1 per cent) agar with a pH of 7.7 is to be preferred. For tubercle bacilli, a glycerin agar may be employed since an egg medium may be unsatisfactory because heating for inspissation and sterilization may break up some disinfectants. For parasitic molds like *Trichophyton rosaceum*, *Microsporon audouini* and *Achorion schoenleinii*, Sabouraud's maltose medium titrated to + 1.0 to phenolphthalein, may be employed. What-

ever medium is chosen, it must be adopted for cultivating the test organism, should be sterile, liquefied by heating, and added to the tubes containing varying dilutions of the disinfectant while still fluid after being cooled to 40 to 45° C. After hardening has occurred, each tube is inoculated in as uniform manner as possible and the tubes incubated for a period of 5 to 10 days (mycostatic tests for 2 weeks or longer at room temperature) as decided upon. The results are expressed according to the highest dilution of disinfectant capable of preventing the growth of the test organism. Mercuric chloride or phenol controls may be included and the results expressed in terms of coefficients.

# DIAGNOSTIC MYCOLOGICAL METHODS

## PRINCIPLES

1. The majority of diseases due to the pathogenic fungi and yeasts with which the clinician comes in contact are those which produce superficial lesions of the skin and mucous membranes. The former are caused, for the most part, by members of the genera *Achorion*, *Microsporon*, *Trichophyton*, and *Epidermophyton*. The most important subclass consists of the Typhomycetes, or fungi imperfecti, which include practically all the fungi pathogenic to human beings.

2. In Sabouraud's classification, fungi having small spores in which the elements are found in mosaic arrangement and in profusion on the surfaces of hairs, are known as *Microspora*. The next group consists of the *Trichophyta*, divided into endothrix, which invades the hair shaft with the formation of large spores in linear arrangement, and ectothrix, which forms chains of spores external to the hair. The endothrix microorganisms are usually not inoculable into laboratory animals whereas the ectothrix fungi are often pathogenic for them. The genus *Achorion* includes only one common pathogen, namely, *Achorion schoenleinii* the cause of favus. The term "Epidermophyton" indicates lack of invasion of a hair follicle.

3. Laboratory diagnosis can usually be made by direct microscopic examinations of hairs or scrapings from the lesions. Not infrequently, however, cultures are required, as in differentiation between *Microsporon audouini* and *Microsporon lanosum*. Clinical differentiation is based upon the more inflammatory type of lesion produced by *Microsporon lanosum*, but noninflammatory lesions may be produced by this fungus. In such cases identification of species is necessary for correct therapeutic measures. Thus lesions due to *Microsporon lanosum* usually respond readily to the local application of fungicides, whereas those due to *Microsporon audouini* can be treated successfully only after epilation.

4. Less readily recognized clinically are those mycotic diseases which either occur less frequently or produce infections without an initial local lesion. Examples are pulmonary and intestinal actinomycosis, chromoblastomycosis, systemic histoplasmosis and torula meningitis. Indeed, diagnosis of such infections is often delayed, or in the case of a fatal termination, discovered only at autopsy.

5. Laboratory diagnosis is based upon various examinations. Direct microscopic examinations of specimens of skin scrapings, hairs, nail scrapings, pus or exudates are the simplest and the first step in establishing diagnosis, but rarely permit one to identify species. Cultural methods, including hanging-drop or slide cultures, are frequently the only means of identification. The phenomenon of fluorescence is helpful in determining the presence of tinea capitis or of tinea versicolor. Animal inoculation tests are sometimes helpful and especially in the case of deep fungus infections if negative results are observed with direct examinations or cultures.

## COLLECTION OF MATERIAL

1. The selection of suitable materials for examination is very important. If there are different types of lesions, specimens should be obtained from all. An abundance of material is usually desirable, but a small amount of good material is better than a large amount of unselected material. Specimens should be collected in sterile containers for delivery to the laboratory, but it is frequently better to send the patient

for the best possible selection of material and to reduce the incidence of contaminations. Specimens for examination should be sent to the laboratory and examined without delay. Material more than 3 or 4 hours old is less apt to prove satisfactory. Whenever possible specimens should be taken by or in the presence of the examiner.

2. Since treatment may affect the abundance and the stage of development of a fungus, material from untreated areas and especially recent ones, is preferred. Thus, the components of a medicament may obscure a fungus or confuse the examiner by their similarity to fungi; *i.e.*, oil droplets may resemble yeast cells. With ringworm of the scalp the infected hairs should be selected while the patient is observed under filtered ultraviolet rays since these reveal the sites of *tinea vesicolor* which are not readily discerned in daylight.

3. Removal of accidental saprophytic fungi from the surface of a lesion may usually be accomplished by cleansing with 70 per cent alcohol. Sterile instruments should be employed for the collection of material.

4. When infection produces a porous condition of the nails, the deeper parts are preferred for examination. Small portions of crumbly material are better than large clippings or even the entire nail. Horny or scaly material is practically useless for direct examinations, but suitable for cultures. Fungi are frequently found at the sites of apparently healed lesions which are sometimes responsible for recurring infections unless treatment is continued.

5. In the case of pustules or abscess-like lesions it is advisable to aspirate the exudate from unopened lesions with a sterile syringe.

6. Biopsies for mycologic examination should be taken aseptically and sent to the laboratory in sterile containers. Part of the specimen should be fixed and sectioned for microscopical examination and the remainder used for mycological and bacteriological examinations.

7. In suspected mycosis of the lungs, a specimen of sputum or a fragment of tissue obtained bronchoscopically should be examined. It is frequently difficult to decide by direct or cultural examinations whether *Monilias*, *Actinomycetes* or other microorganisms are actually producing infection of the lungs or are merely present in the mouth as saprophytes.

8. Feces specimens should be collected as for bacteriological examinations and sent to the laboratory in sterile containers.

9. With every specimen, in addition to the usual data, the following information should be furnished: (*a*) the nature and source of the material, (*b*) the time of collection, and (*c*) the type of fungus suspected by the clinician.

10. Material for mycological examination should be divided into three parts: (*a*) for direct examination, (*b*) for culture, (*c*) for possible animal inoculation and special tests.

### GLOSSARY OF COMMON MYCOLOGIC TERMS \*

**Thallus:** The actively growing, vegetative organism as distinguished from the reproductive portions.

**Hypha:** The single thread-like portion (Fig. 215).

**Mycelium:** A group or matted mass of branching hyphæ.

\* After Simmons, *Laboratory Methods of the United States Army*, Lea & Febiger, Philadelphia.

**Septa:** Divisions of a hypha formed by transverse partitions.

**Spores:** Cells developed for the propagation of the species.

**Conidia:** Spores formed directly from the vegetative portion by abstriction, budding or septate division.

**Conidiophore:** The hypha bearing a spore or group of spores.

**Ascospores:** Group of spores, usually 4 or 8, enclosed in a sac, or ascus.

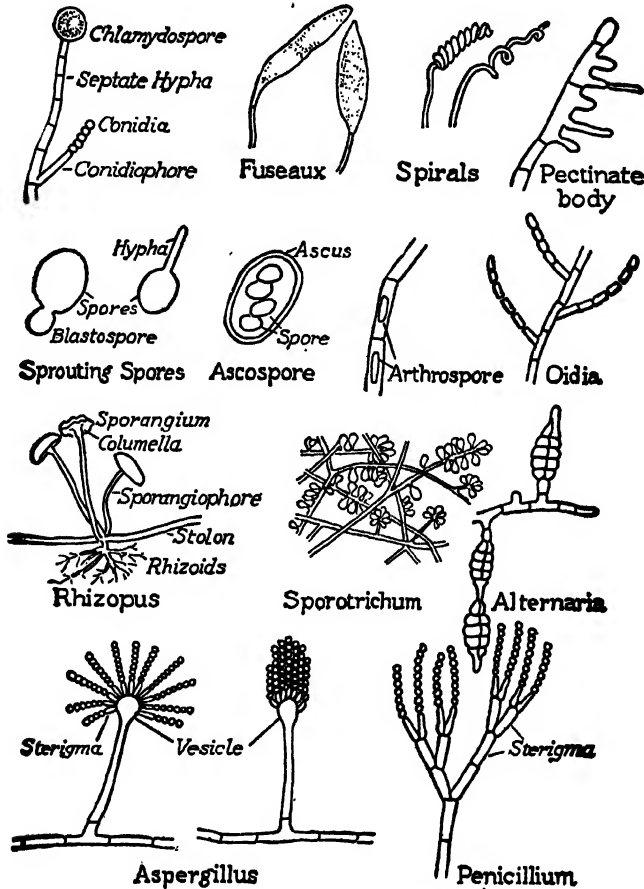


FIG. 215.—TERMINOLOGY USED IN THE DESCRIPTION OF FUNGI

(From Simmons *Laboratory Methods of the United States Army*, Lea and Febiger, Philadelphia.)

**Oospores and Zygosporos:** The spore resulting from the union of two similar spores is a zygosporos; if the spores uniting are male (antheridium) and female oosporangium) the resultant spore is an oospore.

**Endospore:** A spore formed within an outer envelope.

**Blastospore:** A spore formed by budding.

**Arthrospore:** A spore formed of segments of a hypha and released by disarticulation.

**Oidia:** Arthrospores of cylindrical form.



*Chlamydospore*: A large spore, either intercalary or terminal, with tough and frequently double contoured (thick) wall, undergoing encystment.

*Thallospore*: Any spore formed from the main hypha (or thallus) directly, as in the preceding three.

*Sterigma*: A short stalk bearing chains of conidia (as in *Aspergillus*).

*Vesicle*: The swollen end of a hypha bearing groups of spores.

*Columella*: The distal end of a hypha forming the supporting center of a sporangium.

*Coremium*: Bunched groups of conidiophores seen in some species. (Resemble bunch of asparagus.)

*Sporangium*: A sac containing an indefinite number of spores, usually many, at the end of a hypha.

*Sporangiophore*: A hypha bearing a sporangium.

*Stolon*: Runner-like branches of certain fungi (*Rhizopus*).

*Thyrses*: Groups of conidia which are formed along the sides of an unbranched terminal hypha.

*Grappes*: Large groups borne or branched conidiophore clusters.

*Rhizoids*: Root-like groups occurring along stolons.

*Fuseaux*: Fusiform septate spores, produced by certain fungi (*Trichophyton*).

*Spirals*: Terminal coils seen in some species.

*Pectinate bodies*: Comb-like structures formed by some fungi.

### DIRECT METHODS OF EXAMINATION

The purpose is to determine the presence of fungus material. In many instances this is sufficient for diagnostic purposes and in the case of tinea versicolor and a few other mycoses, the sole means of examination. A positive result is much more valuable than a negative one. Direct examinations, however, do not usually suffice for establishing the identity of the species.

1. Place some hairs, scrapings, scales, etc., on one end of a clean slide and add a small drop of 10 per cent solution of potassium or sodium hydroxide. Place on a coverglass and add almost enough hydroxide to fill in the space between the coverglass and the slide. More concentrated solutions of hydroxide may result in crystallization and interfere with the examination.

2. Allow the hydroxide to act for 20 to 30 minutes or until the tissue elements have been dissolved, thus leaving the fungi free for observation. Pass the slide through the flame of a Bunsen Burner three or four times. Examine it under the microscope. If the preparation is not clear, reheat it and examine. Repeat this until the tissue is clear enough for a satisfactory examination.

3. Examine microscopically by reduced transmitted light, under low and high magnification, for the presence of fungi.

4. Observe the presence of molds within the hair or outside the hair, or both within and without.

5. A solvent which may be used when time can be allowed for clearing of the material, and which offers a semipermanent specimen, is an aqueous solution containing 5 per cent potassium hydroxide and 25 per cent glycerin (Lewis and Hopper). With this, little or no crystallization occurs and the material does not dry. In the examination of pus, when for instance actinomycosis or blastomycosis is suspected, this

solution is advantageous as a solvent since the pus cells are destroyed and the fungus material becomes more apparent.

6. Staining is seldom practical in the superficial mycoses because the hydroxide tends to decolorize. Probably the best stain is that described by Linder and modified by Henrici called "lactophenol" and prepared by dissolving 20 gm. phenol crystals in 20 cc. distilled water, 20 cc. lactic acid syrup and 40 cc. glycerin; after solution is complete add 0.05 gm. cotton glue (aniline blue, W. S., C. I. No. 707) with gentle heating.

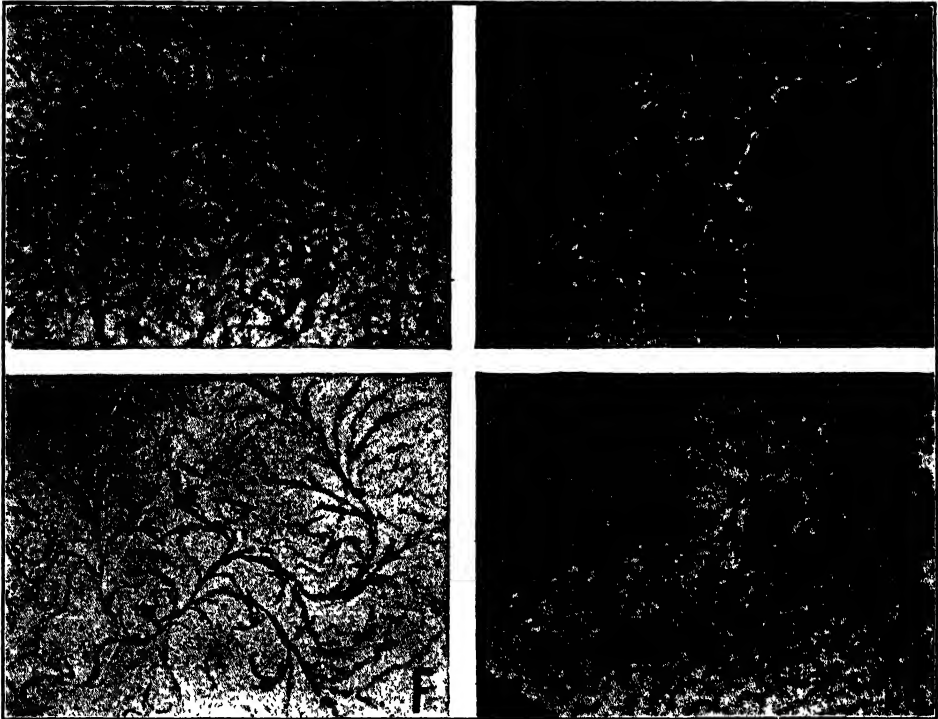


FIG. 216.—ARTEFACTS FREQUENTLY FOUND IN SKIN SCRAPINGS

A, cotton fiber; D, mosaic fungus; F, crystals of potassium hydroxide; G, oil globules, which may simulate fungus spores. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

7. Thin scales, such as those of tinea versicolor, erythrasma or pityriasis capitis, may be placed on a slide, washed in acetone to remove fat and mixed for 3 minutes with Löffler's methylene blue, which is then drawn off with blotting paper. The specimen is then dehydrated with 95 per cent alcohol and xylene and mounted in Canada turpentine. This method, described by Lewis and Hopper, gives a permanent stained mount, but is not suitable for thick sections.

8. Filaments are rarely observed in *hairs* except in favus (*Achorion schoenleinii*). Spores vary in size, being largest in the endothrix Trichophyta (*T. violaceum*, *T. crateriforme*, *T. sulfureum*).

9. In *scales* from tinea glabrosa, filaments are usually present. If the disease is of

long duration, spores may be found. In *tinea versicolor*, groups of double-contoured spores are observed with numerous segmented filaments. In *tinea cruris*, filaments or spores in chains are seen. These are large in the case of *E. inguinale* and likewise more numerous than in the case of infections due to *T. purpureum* or *T. gypseum*.

10. In preparations of *macerated skin* the appearance of *T. gypseum*, *T. purpureum* or *E. inguinale* is similar to that observed in scales. *Monilia albicans* is sometimes revealed as clusters of spores and nonseptated hyphae.

11. In preparations of *nails* the fungi are found on the surface in leukonychia trichophytica. In infections due to *T. gypseum* or *T. purpureum* the fungi are often found in the deeper parts. The hyphae seldom branch and do not occur in a tangled network. Many spores may be present.

12. Fungi are rarely found in material from *superficial pustules* or *blebs*. Granules usually denote actinomycosis. *Sporotrichum schenki* is difficult to find, but budding *Blastomyces* and the endospores of *Coccidioides* may be found.

13. The direct examination of *sputum* is not reliable except in the case of *C. immitis*. Contamination is hard to eliminate.

14. *Artefacts* commonly occur and may readily result in error. These include the so-called mosaic fungus (Weidman), the exact nature of which is in dispute, saprophytes, oil and grease, air, cotton fibers, feathers and many other substances (Fig. 216).

### CULTURAL METHODS OF EXAMINATION

1. The area of skin from which material is to be taken should be cleansed with 70 per cent alcohol. It is then scraped with the blade of a sterile scalpel. Hair is best removed by epilating forceps. Scrapings of the tongue give a good specimen from the mouth. Transferring the material immediately from the patient to the culture medium gives a high percentage of cultures free of contamination.

2. If the material consists of dry scrapings, soak for an hour in 70 per cent alcohol known to be free from spores and molds.

3. Inoculate 4 tubes of Sabouraud's dextrose agar. Inoculate each slant at 3 points, slightly breaking the surface of the medium to introduce spores.

4. Place some tubes in the incubator (37° C.) and allow others to grow at room temperature. It is advisable to paraffin the cotton stoppers to prevent evaporation of the medium.

5. Several tubes of 4 per cent dextrose or maltose broth (pH 5.2) may be inoculated at the same time and incubated in the same manner.

6. Observe the cultures daily, but do not open the tubes unless definite growth is seen. If a colony is at prime in 7 to 10 days it may be considered a fast grower; if 3 weeks elapse before it can be recognized, it is a slow grower; most fungi are of intermediate character. One fungus may cover the entire surface of an agar slant within 2 weeks; another fungus, such as *A. schoenleinii*, never covers more than a small portion.

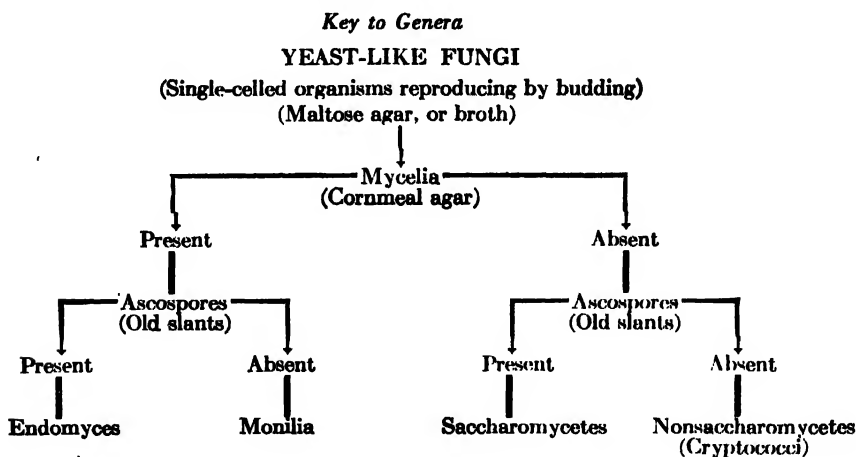
7. When growth occurs, observe characteristics of the colony. A colony may be flat, rounded, fissured, cerebriform, umbilicated, folded or concentrically ringed. Changes in the composition of the medium may affect its gross appearance. The margins may be sharply defined or may fade into the medium. A downy or filamentous growth occurs when the vegetative aerial mycelia predominates and is loosely arranged.

If the mycelia are closer together, the growth appears compact or velvety. A granular surface is due to the presence of spores. A pasty surface denotes a yeast-like organism, and a waxy appearance is characteristic of *A. schoenleinii*. A violet hue is characteristic of *T. violaceum*. The typical port-wine color of *T. purpureum* rarely appears in the growth until after 2 or 3 weeks. A brownish discoloration of the medium is observed with many different fungi. Many lose their colors after repeated subcultures. The character of the culture medium is important. Cultivation on potato-carrot agar will sometimes bring out pigments not otherwise produced. Submergence of the colony is seen in cultures of *A. schoenleinii* and of several other fungi. It is difficult to obtain characteristic colonies of *T. violaceum* and *T. crateriforme* during the winter months; *T. purpureum* grows well, but shows considerable variation during the summer months.

8. Examine wet or stained preparations microscopically.

9. Subculture on cornmeal agar and honey agar slants or plates and cultivate at room temperature or in the incubator for 48 hours or longer.

10. At this stage of examination of fungi having a yeast-like form in primary cultures on Sabouraud's agar or broth, it is possible to distinguish the following genera based upon the presence or absence of mycelial filaments (on cornmeal agar) and the presence or absence of asci (on old cultures):



11. Ascospores may be demonstrated in old cultures (at least 10 days old) of the fungus on Sabouraud's or cornmeal agar by preparing smears from the dried top of the growth and staining (Beauverie) as follows: (a) Fix with heat; (b) cover with carbolfuchsin and gently steam for 2 minutes; (c) decolorize with 25 per cent glacial acetic acid; (d) counterstain with Löffler's methylene blue for  $\frac{1}{2}$  minute; (e) wash with water, dry and examine. Ascospores are red, vegetative cells blue.

12. In order to identify species of fungi, it is often necessary to study the character of their spores and their arrangement. These are characteristically formed in the aerial portions of the colony. If portions of a colony are removed and mounted there results great disruption in the arrangement of the material. For this reason the cover-slip method of examination by Henrici may be employed as follows: (a) Moistened filter paper or a damp blotter is placed on the bottom of a Petri dish and sterilized in an autoclave at 121° C. for 15 to 20 minutes; (b) six coverglasses are cleaned, flamed

and placed on the moist blotter or filter paper; (c) a special agar (2 per cent dextrose, 0.5 per cent peptone and 2 per cent agar) is melted, cooled to 40 to 50° C., inoculated with spores of the growth and well shaken; (d) a thin layer of the inoculated agar is spread over the surface of the coverglasses; (e) these are incubated in the Petri dish at room temperature; (f) when sufficient growth has taken place, the coverglasses are removed with flamed forceps, each inverted on drops of water on slides and examined immediately.

13. The Brown micro slide (Fig. 217) method for fungus culture is likewise very satisfactory as follows: The slide is sterilized by passing it through a flame. If agar



FIG. 217.—BROWN MICRO SLIDE FOR FUNGUS CULTURE

is to be used, the slide should be placed on a warm stage or on a hot water bottle. Small droplets of mineral oil are placed at the six points marked "x". A large loop of the inoculated medium (for example, Sabouraud's agar) is placed on the central stage at the spot marked "O". The preparation is then carefully covered with a flamed 25 mm. coverglass which should cause the inoculated medium to spread out in the form of a broken circle on either side of the notch at the edge of the stage, which notch serves as an air inlet. The slide may now be incubated in a Petri dish containing a piece of moist filter paper to prevent drying. If it is desired to arrest growth of the culture at any stage and to make a permanent preparation, place the slide culture into a desiccator over formaldehyde solution for several hours and then seal off the exterior air inlet, *i.e.*, short, straight moat extending from edge of slide to outer concentric moat, with paraffin by means of a hot spatula. The culture can be observed at any time under the dry objectives of the microscope. Structures near the coverglass can be observed under the oil immersion objective.

14. Additional cultural studies on various sugar media with pH 5.2 (maltose, saccharose, lactose, levulose, mannose, galactose, glycerol, trehalose, dextrin, inulin) may be required as well as on gelatin and special media.

15. After prolonged cultivation, many fungi assume a vegetative character, as shown by a white fluffy growth almost always starting at the point of inoculation. Within a short time the entire colony may be covered. Some fungi, such as *M. lanosum*, develop this character after a short time, while other fungi, such as *T. violaceum*, never assume the vegetative form. Subculturing sometimes causes the fungus to assume its original nature, but in such instances the growth is probably not truly pleomorphic.

## ANIMAL INOCULATION METHODS OF EXAMINATION

1. In superficial infections animal inoculation tests are seldom resorted to for diagnostic purposes. In the deep or systemic mycoses they are advisable and sometimes necessary.

2. According to Weidman, mice and rats are the animals of choice. Guinea-pigs

and rabbits may be employed. Inoculations may be cutaneous, intraperitoneal or testicular. Cats (especially kittens) may be used for cutaneous inoculation.

3. The inoculum is usually a saline suspension freshly prepared from a growth on Sabouraud's agar or from finely ground fresh tissue.

4. When a "take" occurs after inoculation of a fungus which causes a deep or systemic infection in human beings, a comparable infection is looked for in the experimental animal. If it dies spontaneously, postmortem examination will likely show the degree and character of the infection. If the animal is still living 6 or 8 weeks after inoculation, it may be killed and examined.

### METHODS FOR THE IDENTIFICATION OF *MICROSPORUM AUDOUINI*

1. This fungus is the usual cause of ringworm of the scalp (*Tinea capitis*).

2. Upon direct examination it appears in the form of a mosaic sheath around stubby hairs (Fig. 218). There is little tendency to chain formation. The spores are round and small. In infections of the glabrous skin, mycelia may be detected. Lanugo hairs are occasionally infected.

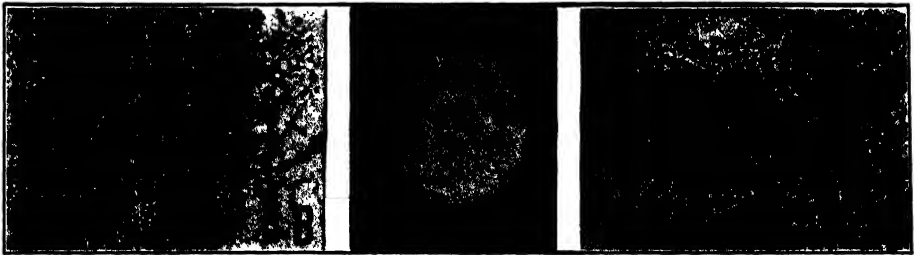


FIG. 218.—*MICROSPORUM AUDOUINI*

*B*, infected hair showing mantle of spores ( $\times 365$ ); *D*, cultural growth after two weeks; *F*, culture mount showing microconidia in clusters and as hyphae sporiferae. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

3. Colonies begin as a white feathery fluff and develop moderately into a grayish-white fluffy growth. Aerial growth is scanty. There is usually a central elevation. Pleomorphism is uncommon.

4. Microscopically, cultures show only a few rudimentary fuseaux but are characterized by peculiar structures known as "pectinate bodies". These are small parallel protuberances extending out from one side of swollen hyphal branches resembling a comb. Other features are the presence of chlamydospores, racket mycelia and microconidia.

### METHODS FOR THE IDENTIFICATION OF *MICROSPORUM LANOSUM*

1. This fungus produces ringworm of the scalp (*Tinea capitis*), a rare ringworm of the bearded region (*Tinea barbae*) and ringworm of the skin (*T. glabrosa*) as well as interigo of the toes.

2. Upon direct examination its appearance in the sheath around the infected hair

is indistinguishable from that of *M. audouini* in the same location (Fig. 219). The individual spores are small and round and are present in clusters. On the smooth skin, mycelium is present in small amounts. Lanugo hair is sometimes infected.



FIG. 219.—*MICROSPORUM LANOSUM*

*B*, infected hair showing mosaic of spores outside the hair shaft ( $\times 350$ ); *E*, growth on dextrose agar after 10 days; *J*, culture mount showing pointed fuseaux. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

3. Growth is moderately fast. A downy fluff appears around which is yellowish pigment. In 2 weeks the central part of the colony is depressed. The aerial growth is abundant, wooly and buff-tan in color. Grooves, if present, are often concentric although radial grooves are common.

4. Material taken from the surface of the culture contains numerous lenticular fuseaux, which are thick-walled and provided with spines. Microconidia are also produced along the sides of the mycelium. Chlamydospores occur and are found with thick walls.

## METHODS FOR THE IDENTIFICATION OF *ACHORION SCHOEENLEINII*

1. This fungus is the cause of favus. It may also affect the smooth skin (*Tinea glabrosa*) and the nails. Diseased hairs are surrounded by yellowish, waxy crusts at their bases called "scutula".

2. Upon direct examination, large spores in chains may be found within the hair



FIG. 220.—*ACHORION SCHOEENLEINII*

*C*, irregular filaments and spores in the hair shaft accompanied by air bubbles ( $\times 325$ ); *E*, colony after one month; *H*, culture mount. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

substance (Fig. 220). Air spaces may also be noted in the hair and attached to it; this is always suspicious. If a scutulum is examined, a mass of sporulated hyphae will be found. Material from lesions of the skin show but few hyphae. Material from infected nails shows chains of spores.

3. Growth in cultures is slow; sometimes a recognizable primary colony does not develop before 3 weeks. The growth is compact and smooth and presents a characteristic waxy appearance. The colony grows down into the medium and in time produces cracking of the agar.

4. Microscopic examinations show numerous club-shaped terminal branches, many of which are notched at the tips. A characteristic formation is the presence of clusters of such clubs. Chlamydospores in large numbers may be observed also.

### METHODS FOR THE IDENTIFICATION OF TRICHOPHYTON GYPSEUM

1. This fungus and its variants (*T. purpureum* and *E. inguinale*) are the chief causes of intertriginous infections (Tinea pedis or "athlete's foot", Tinea cruris, Tinea manuum, Tinea unguium). It may also produce ringworm of the skin (Tinea glabrosa) and rarely of the bearded region (Tinea barbae). It is unstable and always likely to produce variants. It is possible that *Trichophyton interdigitale* (Kaufmann-Wolf) is one of these.

2. Upon direct examinations it is found external to the hair (ectothrix Trichophyton) with chains of small spores (Fig. 221). In scales, macerated skin and nail tissue the organisms appear as chains of spores or as segmented mycelium with little branching.

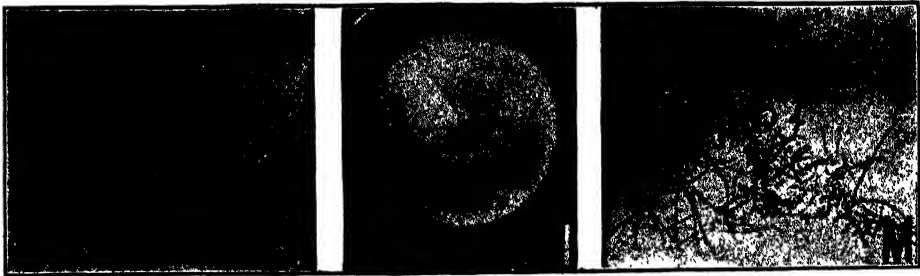


FIG. 221.—TRICHOPHYTON GYPSEUM

A, fungus in direct mount from the skin ( $\times 150$ ); I, usual type of colony; M, culture mount ( $\times 150$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

3. In cultures the fungus first appears as a white and fluffy growth. Later it becomes velvety, flat and light buff or buff-yellow in color, with a boss at the center. It may also occur as a granular or powdery type of growth with fluffy changes as it ages with a light buff or maize-yellow color. Its variant, *Trichophyton interdigitale*, begins as a downy feather-like growth which develops rapidly with many aerial hyphae; it is almost pure white. Another variant (*Trichophyton niveum*) begins as a white and fluffy growth which later becomes compact with irregular elevations and depressions.



4. Upon microscopical examination the ordinary type is characterized by spirals. Fuseaux with blunt ends are present in small numbers. Nodular organs, pectinate bodies, racquet mycelium and chlamydospores are usually found. The mycelium is septate and usually branched. Microconidia occur as thyrsi and as *grappes*.

### METHODS FOR THE IDENTIFICATION OF TRICHOPHYTON VIOLACEUM

1. This endothrix Trichophyton causes a type of ringworm of the scalp (*Tinea capitis*) which is exceedingly refractory to treatment. It may also attack the bearded region, producing a type of *Tinea barbae*.

2. In direct examinations it is found invading the shafts of hairs (Fig. 222). The spores are larger than those of the microspora and are arranged in rows or beads.

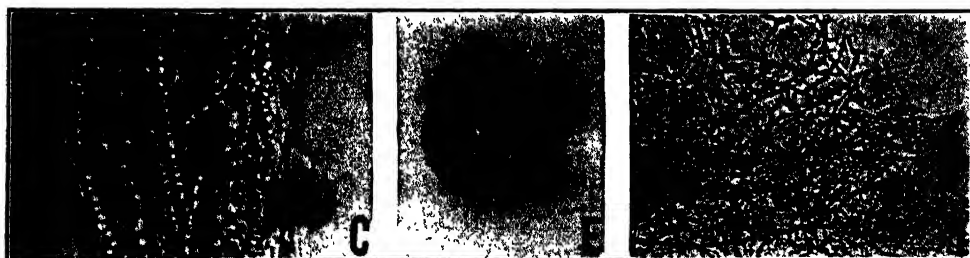


FIG. 222.—TRICHOPHYTON VIOLACEUM

C, infected hair showing involvement of the shaft with large spores arranged in chains ( $\times 355$ ); E, typical colony after four weeks; I, culture mount showing simple filaments and no specialized forms. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

In scales of skin and in nails, the fungus also occurs in the form of sporulated mycelium.

3. Growth is slow in cultures. The colony is small, well defined, smooth, shiny and compact with a deep violet color. The surface is convoluted with radial grooves near the periphery.

4. Upon microscopical examination no free conidia or thyrsi are found. Mycelia are short with numerous septa. Many irregular and bizarre branches are present. Chlamydospores are numerous in older cultures.

### METHODS FOR THE IDENTIFICATION OF TRICHOPHYTON CRATERIFORME

1. This fungus is sometimes a cause of ringworm of the scalp (*Tinea capitis*); it may also be found in ringworm of the smooth skin and nails.

2. Upon direct examination the hair shafts show the presence of large spores in chains (Fig. 223). At times the hair appears to be entirely filled with fungus elements.

3. In cultures the fungus grows slowly. The surface of the growth is creamy-white, compact and velvety. The central portion is sharply crateriform and yellowish.

4. Upon microscopical examination small conidia are observed either in short

stocks or coming directly off hyphae and sometimes in clusters. Chlamydospores are common.

### METHODS FOR THE IDENTIFICATION OF TRICHOPHYTON PURPUREUM

1. This fungus frequently infects the interdigital webs of the toes and sides of the feet (*Tinea pedis* or "athlete's foot"), the inner surfaces of the thighs (*Tinea cruris*) and the hands and nails (*Tinea manuum* and *Tinea unguium*).

2. Upon direct examinations so few fungus filaments occur that repeated examinations may be required before they are found (Fig. 224).

3. In cultures the growth at first is fluffy and pure white. Later it becomes almost velvety and radial grooves may appear. The back of the colony soon develops a typical rose-purple color which gradually spreads.

4. In cultures there are many sterile hyphae. Some microconidia may be seen, as likewise racquet mycelia. Fuseaux and chlamydospores are infrequent.

### METHODS FOR THE IDENTIFICATION OF EPIDERMOPHYTON INGUINALE

1. This fungus is the usual cause of *Tinea cruris* and is capable of producing epidemics in institutions, in camps, on ships and elsewhere.

2. In direct examinations of scales of skin large numbers of the fungi are usually observed as chains of spores which tend to be flattened (Fig. 225).

3. In cultures a growth may not be observed for 2 or 3 weeks. The colony has a velvety or felted surface with irregular folds and grooves; aerial hyphae are sparse. The color is characteristically grayish olive-drab. Whitish tufts appear early and may eventually cover the entire agar slant.

4. Upon microscopic examination club-shaped fuseaux resembling bunches of bananas are frequently observed with numerous chlamydospores. Racquet mycelium is often seen.

5. *Epidermophyton rubrum* is a closely allied species. *Epidermophyton interdigitale* is

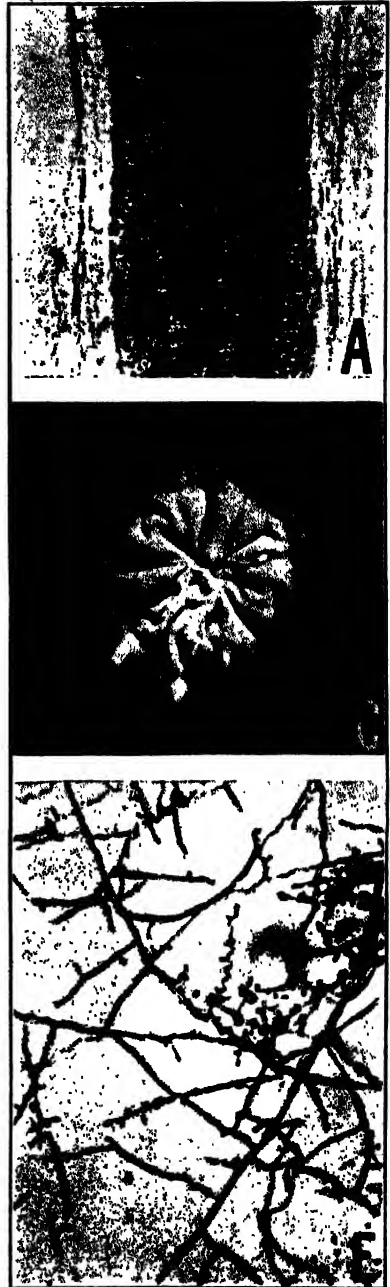


FIG. 223.—*TRICHOPHYTON CRATERIFORME*

A, infected hair showing spores in the shaft ( $\times 210$ ); C, colony after one month in the winter; E, culture mount showing aleurospores ( $\times 210$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)



FIG. 224.—*TRICHOPHYTON PURPUREUM*

*B*, appearance of fungus in a direct preparation from fingernail ( $\times 305$ ); *E*, culture growth; *J*, culture mount showing microconidia and fuseaux. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)



FIG. 225.—*EPIDERMOPHYTON INGUINALE*

*A*, numerous wavy filaments in a direct mount from the scales; *C*, colony after six weeks showing pleomorphic tufts and fringe; *D*, culture mount ( $\times 200$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

probably identical with the white fungus of Kaufmann-Wolf and similar to the *T. niveum* group. The culture is at first pure white, later gold. Some strains show a reddish color. It is made up of tangled hyphae, mostly sterile, but with few scattered aleurospores. Macroconidia may be seen in old cultures.

### METHODS FOR THE IDENTIFICATION OF *MONILIA ALBICANS*

1. This is a pathogenic yeast-like organism of considerable importance in the production of cutaneous, mucocutaneous and systemic infections. Localized lesions of the skin include intertrigo of the toes and of the inframammary, axillary, inguinal, intergluteal, anal and umbilical regions, as well as eczematous patches on the smooth skin, eyelids and scalp. Paronychia and onychia may be produced. Thrush, perlèche and smooth tongue are common conditions caused by this yeast. The intestinal tract of individuals with moniliasis, as well as those without clinical manifestations, may harbor the organism. It is also capable of producing vaginitis. Likewise infections of the bronchi and lungs simulating tuberculosis; meningitis may occur.

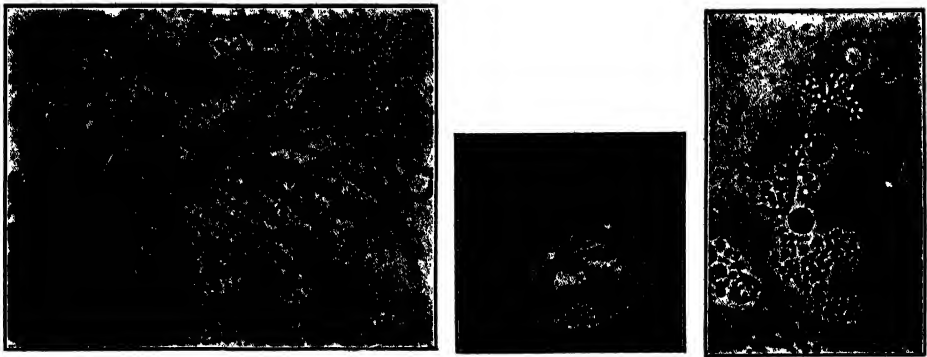


FIG. 226.—*MONILIA ALBICANS*

*B*, appearance of the fungus on direct examination of oral thrush; *E*, giant colony showing pasty growth and multiple craters in the center; *I*, mount from colony showing clusters of budding cells and chlamydospores ( $\times 445$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

2. In direct examinations the organism usually appears as a tangled network of fine mycelium with clusters of spores (Fig. 226). Yeast-like cells are also found laterally along the branches and may lie free (Figs. 227 and 228). In scrapings from nails and many other locations, only a few hyphae or budding cells may be observed, and frequently the results are uncertain. A number of nonpathogenic monilia may occur upon the skin and in the intestinal tract, and must be differentiated; *Monilia candida*, *Monilia parasitosis* and *Monilia krusei* are among the most common of these.

3. The growth on Sabouraud's agar is moderately fast and is first noted after 2 or 3 days. It is wet, pasty and cream colored. The surface is usually smooth except near the center of the colony where it has a honeycombed appearance due to ruptured air bubbles. On cornmeal agar, a deep stab with a needle containing the yeast results in the characteristic picture of an inverted pine tree. On this medium hyphae are

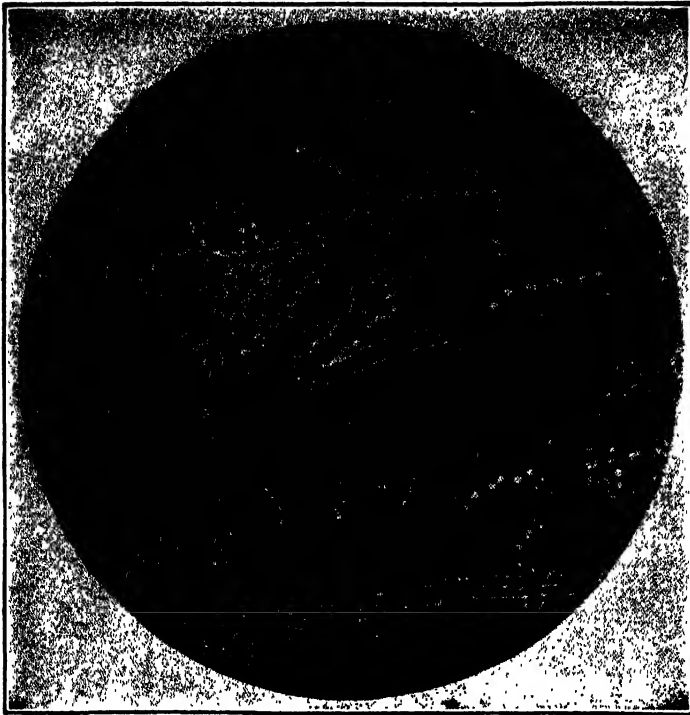


FIG. 227.—*MONILIA ALBICANS*, UNSTAINED, SHOWING MYCELIUM SPORES AND YEAST-LIKE CELLS  
(From Benham)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

developed early, showing clumps of yeast-like budding forms occurring at points of puncture, together with large ball-like clusters. In young cultures many yeast-like oval cells 5 to 6 micra in diameter, and highly granular, showing budding at one or both ends, are commonly observed. It is notable that pure cultures are the rule because *M. albicans* seems to inhibit other microorganisms. Cryptococci do not develop mycelium and other fungi in the monilia group may be distinguished from *M. albicans* by the absence of chlamydospores when they are grown on cornmeal agar.



FIG. 228.—*MONILIA ALBICANS*  
(After Zettnow)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

4. Animal inoculation tests are sometimes of diagnostic value. Intracutaneous inoculation of guinea-pigs causes mild inflammatory reactions. Intravenous injections of rabbits with 1 cc. of 1:1000 suspensions of cultures, usually produce miliary abscesses in the viscera, serous membranes, and kidneys in 4 or 5 days (Benham).

### METHODS FOR THE IDENTIFICATION OF MALASSEZIA FURFUR (MICROSPORON FURFUR)

1. This fungus is the cause of Tinea versicolor; it may also be the cause of Tinea flava and Achromia parasitaria in some cases.

2. Upon direct examinations of scales of skin there is usually an abundance of fragmented mycelia occurring as wavy threads of moderate length with clusters or groups of spherical or ovoid refractile spores apparently with double-contoured walls (Fig. 229). Branching may be observed. The filaments are readily broken up when



FIG. 229.—MALASSEZIA (MICROSPORIUM) FURFUR

Appearance on direct examination of scales showing short filaments and groups of spores with double-contoured walls ( $\times 415$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

one is making the mount. When scales are examined from patches of pseudo-achromia, only a few spores without appreciable grouping may be found, along with a few short filaments.

3. Cultures are not employed as there is no general agreement on the successful cultivation of this organism.

### METHODS FOR THE IDENTIFICATION OF ACTINOMYCES MINUTISSIMUS (MICROSPORUM MINUTISSIMUM)

1. This organism is the cause of erythrasma.

2. Direct examinations of scales are best made with staining as the fungus elements are too small to be seen under the usual low power magnification. Under high power, fine threads may be observed which, upon examination with the oil immersion lens, appear long, tortuous and interlacing (Fig. 230); segmentation may be noted. A few round spores may be found. Granules are occasionally seen. The organism is so

small that it may be mistaken for an accidental bacterial contaminant. No other species of fungus can be confused with it.

3. Cultures are not employed as the successful cultivation of the fungus has not been proven.

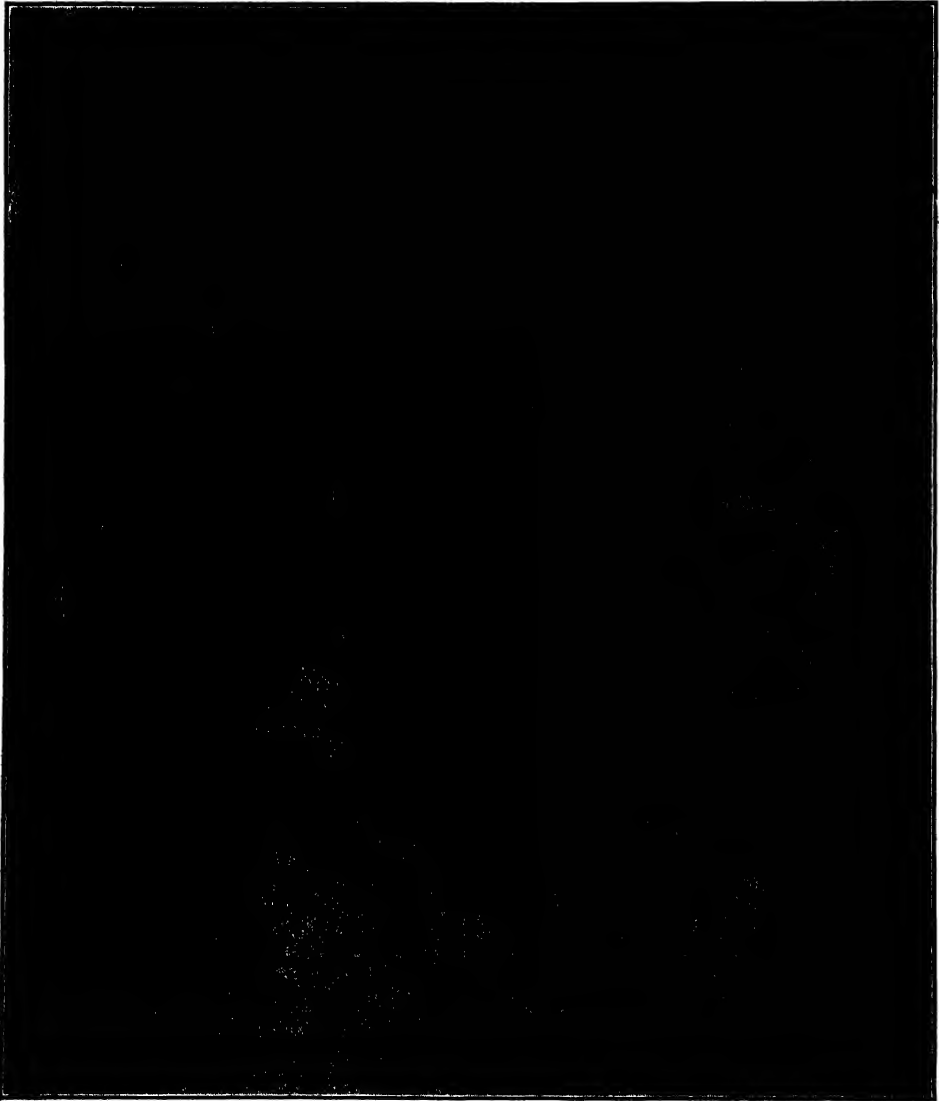


FIG. 230.—*ACTINOMYCES MINUTISSIMUS* (*MICROSPORUM MINUTISSIMUM*)

Stained slide showing numerous interlacing filaments ( $\times 2080$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

**METHODS FOR THE IDENTIFICATION OF HORMODENDRUM PEDROSOI**

1. This microorganism is one of the 3 fungi established as causes of chromoblastomycosis.

2. Direct examinations are conducted with pus removed from fresh lesions. So-called sclerotic cells (Medlar), which may be septate and usually in groups, are typical (Fig. 231). Small septate filaments may be observed also.

3. Cultures on Sabouraud's agar have the shape of a low cone with a diameter of about 45 mm. at the end of 4 weeks. The surface is covered with a grayish nap. Zonation may appear. From the center to the periphery a typical colony presents concentric zones of olive-black, brownish-olive, olive-black, brownish-olive, olive-gray and gray. After 2 or 3 months the colonies are brown.

4. Microscopical examinations of cultures usually show branched or unbranched conidiophores with brownish-olive spores at the tips and later on the sides as well.

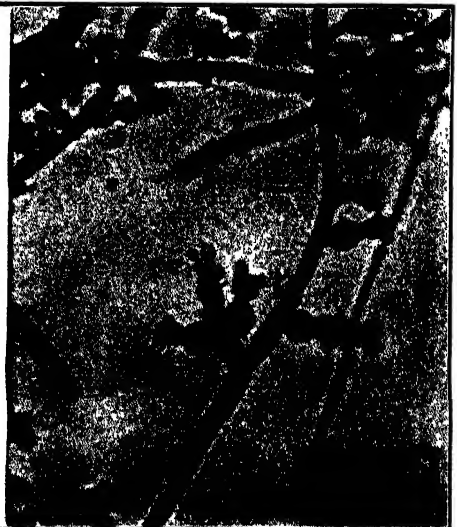
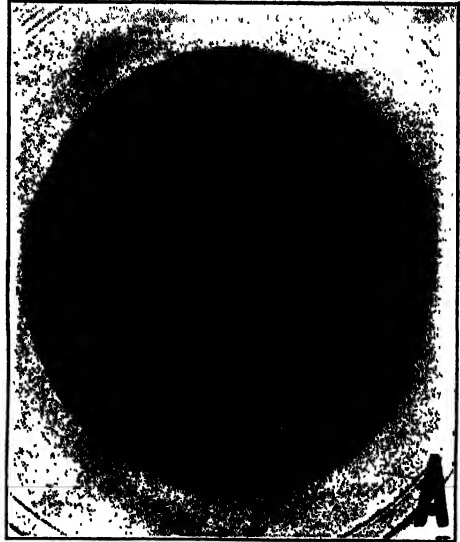


FIG. 231.—HORMODENDRUM PEDROSOI

A, colony after two months; C, culture mount showing the youngest spore at the tip of the chain of spores ( $\times 1000$ ); D, culture mount from an older colony ( $\times 1000$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)



Spores sometimes appear in branching conidial chains; they vary considerably in size and shape.

5. Characteristic lesions are produced by the inoculation of rats.

### METHODS FOR THE IDENTIFICATION OF *ACTINOMYCES BOVIS*

1. This organism is the usual or chief cause of actinomycosis of human beings.

2. Direct examinations are of considerable diagnostic value. If the lesions have broken down, discharges of pus or necrosing tissue may be collected and sent to the laboratory in a sterile container. It is preferable for culturing to aspirate an unopened, softened lesion, which will be free from contaminating bacteria. Remove tissue by biopsy and send to laboratory in a sterile container. In pulmonary actinomycosis, the sputum is examined.

Spread the pus or material in a thin layer over the bottom of a sterile Petri dish. Salt solution may be added if necessary. Examine with the naked eye or hand lens for small grayish or sulphur-yellowish granules, less than a millimeter in diameter. These granules may be colonies of actinomycetes. By means of a platinum loop, place a granule on a slide and cover with a coverglass. Press out gently. If the structural details are obscure, place 2 or 3 drops of 10 per cent sodium or potassium hydroxide on the granule before covering. If the granules are calcified, add a drop of concentrated acetic acid. This will remove the calcium and make possible examination.



FIG. 232.—*ACTINOMYCES* GRANULE CRUSHED BENEATH A COVERGLASS. (After Wright and Brown)

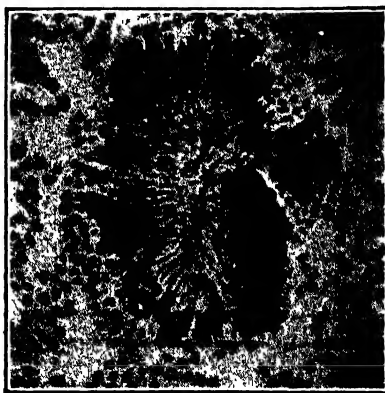


FIG. 233.—*ACTINOMYCOTIC* GRANULE IN PUS, STAINED TO SHOW THE MYCELIUM. (After Kolle and Hetsch)

(From Zinsser and Bayne-Jones, *Text-book of Bacteriology*. D. Appleton-Century Co., New York.)

Examine under the low power of the microscope, 16 mm. and 10 x ocular, with the light somewhat diminished. If the granule is the *Actinomyces bovis*, the center will appear darker and made up of interlacing mycelia, which end in radially arranged terminals (the rays). These rays are closely packed together, are pear- or club-shaped, and are known as "the clubs" (Figs. 232 and 233). There are no spores.

The coverglass may now be removed, the specimen dried and stained by Gram's

method. The central mycelia are gram-positive; the clubs or bulbous ends are gram-negative.

3. Cultures of *Actinomyces* may be misleading as it is frequently present in the buccal cavity as a saprophyte. Secure a number of granules, wash with sterile saline solution and crush. If granules are not found, use portions of the specimen submitted. Inoculate several tubes of dextrose-veal infusion blood agar (pH 7.3 to 7.6). Incubate anaerobically in a jar at 37° C. Later, transplants may grow aerobically. Examine after 4 or 5 days for colonies of the fungus. At first the colonies are small, dry, opaque, white or grayish in color, adherent, and with a somewhat irregularly roughened surface.

Subculture typical colonies to obtain pure culture for use in biologically proving the organism. Prepare smears and stain by Gram's method. The organism is Gram-positive and appears as a number of dichotomously-branched filaments (Fig. 234). "Clubs" are not found. If no growth occurs in 2 or 3 weeks, cultures may be considered negative.

To biologically prove the organism, inoculate duplicate sets of the pure culture on various laboratory media and grow both anaerobically and aerobically in an incubator at 37° C. *Actinomyces bovis* grows in anaerobic environment, the growth being greatest in stab cultures and shake cultures at a depth of 1 to 2 cm. It produces acid and no gas on lactose, maltose, glucose and salicin. Indol shows negative result. Nitrates are reduced. Litmus milk is not changed, gelatin shows slow liquefaction. Löffler's blood serum is not pitted.

4. When cultures are inoculated into guinea-pigs an infection develops in only about 50 per cent. It is usually of minor importance and tends to heal spontaneously. This is interesting in view of the fact that the disease is most likely identical in animals and in human beings.

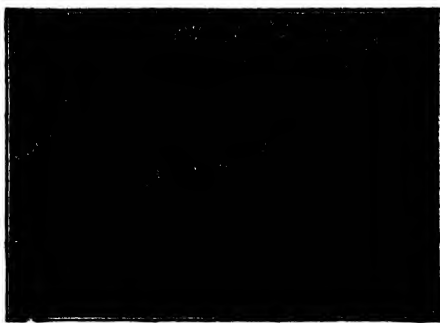


FIG. 234.—BRANCHING FILAMENTS OF  
ACTINOMYCES

(After Wright and Brown)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

## METHODS FOR THE IDENTIFICATION OF SPOROTRICHUM SCHENCKI

1. This organism, which is probably a frequent saprophyte on many kinds of vegetation is the chief, if not the only, cause of sporotrichosis of man in the United States.

2. The organism is present in spore form in pus. If the lesion is not ulcerated, the pus may be withdrawn by aspiration. Some of the pus may be dropped on slides for direct examination, but cultures are more important. In other words, the organism is seldom seen in fresh preparations. When found, it occurs as elongated oval cells, ranging from 3 to 10 micra in length and from 1 to 3 in width. These cigar-shaped spores in the tissues may be found within giant cells (Fig. 235).

3. The organism grows well in most media at room temperature and is readily

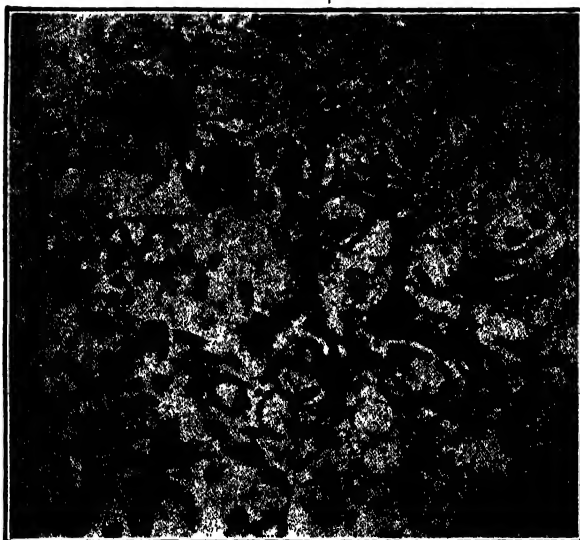


FIG. 235.—SPOROTRICHUM SCHENCKI

Spores in lesion in testis of rat. (From Hopkins)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co. New York.)

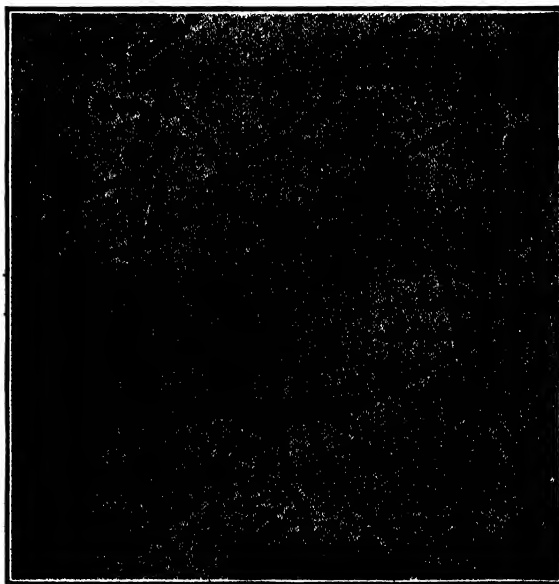


FIG. 236.—SPOROTRICHUM SCHENCKI

Spores in lesion in testis of rat. (From Hopkins)

(From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

identified. Sabouraud's agar is recommended. After 5 to 7 days, there develops moist pinpoint colonies with a fine fringe. These gradually grow and develop a light brown color with irregular central convolutions (Fig. 236). The color gradually turns to dark brown.

4. Upon microscopic examination, the mycelium is profuse, fine and branching. Pear-shaped conidia are present at irregular intervals along the course of the mycelium and may also appear as terminal triads and tetrads. Single spores may also be seen attached directly to the sides of the hyphae. The groups of spores are attached by short stalks (conidiophores).

5. Some of the exudate may be injected subcutaneously or intraperitoneally into male rats. The organisms tend to form localized lesions in the testes and joints. Cutaneous papules may be produced along the tails.

### METHODS FOR THE IDENTIFICATION OF BLASTOMYCES DERMATITIDIS

1. This organism is chiefly concerned in the etiology of blastomycosis (Gilchrist's disease). It usually begins as a local lesion on the exposed parts of the body; sometimes on the tongue. Systemic infections may involve any organ or tissue of the body, especially the lungs, and closely resemble granuloma coccidioides.

2. Direct examinations of pus or sputum usually show the presence of budding thick-walled round or oval granular cells (Fig. 237). If the suspected organisms do not show budding, the coverslide may be rimmed with vaselin. After 24 to 48 hours of incubation at room temperature, budding may be observed.

3. According to Martin and Smith, cultures should be made on both dextrose agar and blood agar. Dextrose agar cultures should be left at room temperature and the blood agar cultures incubated at 37° C. The organism grows readily, but is easily overlooked or lost in cultures due to overgrowth by bacteria. The colonies on blood agar are small, compact and shiny. On dextrose agar they are at first smooth and grayish, but soon become filamentous and white with a central umbo (Fig. 238). With age, the colonies become brown and the surface may crack. Microscopic examinations of dextrose agar cultures show mycelium with lateral conidia and large cells (chlamydo spores). Racquet mycelium may also be noted.

4. According to Spring, the mouse is most susceptible. Intratesticular inoculation is usually employed. The formation of abscesses may be considered as positive. Generalized infections do not always occur.

5. Sections of tissue obtained by biopsy or postmortem should be stained with



FIG. 237.—BLASTOMYCES SHOWING BUDDING FORMS IN PUS FROM A LESION IN MAN. (From Gilchrist)

(From Zinsser and Bayne-Jones, *Text-book of Bacteriology*, D. Appleton-Century Co., New York.)

hematoxylin and eosin, polychrome methylene blue, or theonin. The budding organism may be found in giant cells or in the granulation tissue. The capsule usually remains unstained, the protoplasm is usually basic and the granules deep blue.

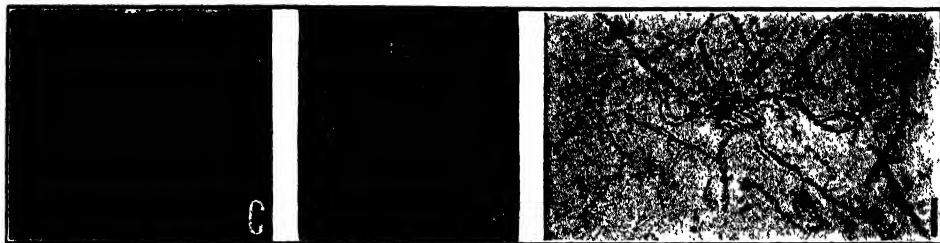


FIG. 238.—BLASTOMYCES DERMATITIDIS

C, histologic section showing the budding microorganism ( $\times 430$ ); F, filamentous colony after three weeks on maltose agar at room temperature; I, hanging-drop preparation of colony grown at room temperature ( $\times 150$ ). (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

## METHODS FOR THE IDENTIFICATION OF COCCIDIODES IMMITIS

1. This fungus is the cause of coccidioidomycosis which is usually due to the inhalation of spores with a primary infection of the lungs. It may also be due to a primary infection of the skin in which trauma occasionally provides a portal of entry for the fungus. Systemic or generalized infection may occur by way of the blood in which almost any tissue or organ may become involved. Infection from man to man is unknown, but laboratory workers may become infected by the inhalation of spores from old cultures.



FIG. 239.—COCCIDIODES IMMITIS

Round and sporulating forms in a giant cell in a lesion in man. (From Rixford and Gilchrist). (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co., New York.)

2. Examinations of free pus and sections of tissue usually show the organism as spheres with double contoured capsules from 5 to 60 or more microns in diameter. Within the organisms are from 6 to 20 spores (Fig. 239). Rupture of the capsule results in their dissemination by way of the blood to other parts of the body. The cytoplasm is granular. No buds are seen, which is the chief point of differentiation from the blastomyces.

3. The organism grows readily on all ordinary media. Streak cultures on Sabouraud's agar, honey agar or cornmeal agar are recommended. Incubate a culture at  $37^{\circ}$  C. and leave a second at room temperature. Within a few days moist colonies appear. A creamy-white fluff soon covers the area which becomes brownish to greenish-black with age. Gelatine shows liquefaction, litmus milk gradually peptonizes, and the sugars are not fermented. Microscopic examinations of cultures show many round forms from 2 to 8 microns in

diameter, from which arise septate and branched typhae. Arthrospores and chlamydo-spores are present.

4. Diagnosis is made with certainty only by animal inoculation. The guinea-pig is usually inoculated subcutaneously or intraperitoneally with pus. This animal is not nearly so susceptible to *Blastomyces dermatitidis*, the organism usually to be distinguished from *Coccidioides immitis*.

### METHODS FOR THE IDENTIFICATION OF *TORULA HISTOLYTICA* (*CRYPTOCOCCUS HOMINIS*)

1. This organism is the cause of torulosis (European blastomycosis). The portal of entry is believed to be the upper respiratory tract. It produces *torula meningitis* which usually runs a prolonged course and is invariably fatal. *Cutaneous torulosis* may occur primarily followed by meningitis or secondarily to meningitis.

2. In centrifugated specimens of spinal fluid the organism may be seen as budding cells of various sizes. India ink preparations usually show wide mucinoid capsules. According to Weidman and Freeman, these should be made quickly with a loopful of India ink on a slide to which is added a loopful of material. After being quickly mixed, a large coverglass is quickly applied and pressed gently (Fig. 240). Capsules may also be observed in stained sections of tissue.

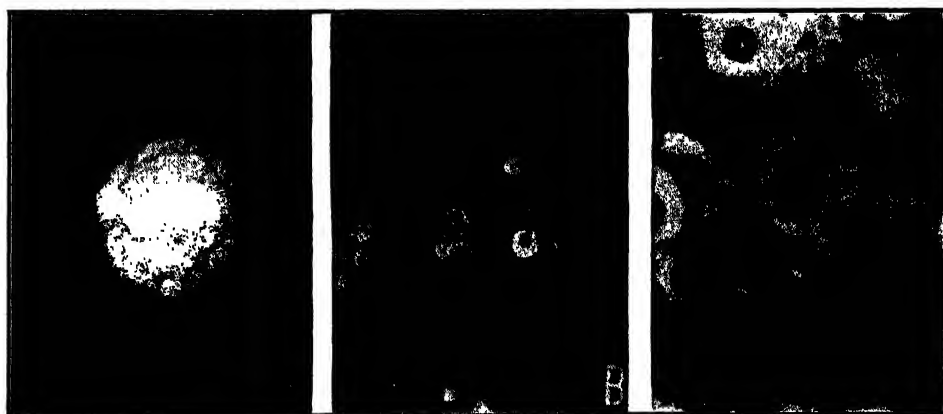


FIG. 240.—*TORULA HISTOLYTICA*

A, colony grown on Sabouraud's maltose agar at 37° C. after two months; B, culture mount in India ink showing budding and thickened capsules; C, histologic sections through the brain of an infected guinea pig, revealing capsulated budding cells. (From Lewis and Hopper, *An Introduction to Medical Mycology*, Year Book Publishers, Inc., Chicago.)

3. The organism grows slowly on Sabouraud's agar. The growth is moist and cream colored, later becoming yellow and then brown. The surface is usually smooth. Upon microscopical examination, mycelium and ascospores are not found. The cells are round or oval, and budding is commonly observed. Preparations made with India ink usually bring out the capsules quite clearly.

4. Animal inoculation tests are advisable. Mice and rats are most susceptible (Benham).

Mycetoma is a suppurating granuloma of the foot, closely related to actinomycosis. The securing of specimen, technic of examination and culture are similar to those described under Actinomycosis.

The granules found in the pus of Madura foot are usually black, although they may be yellowish, greenish or red.

A large number of fungi have been isolated, in addition to 13 species of *Actinomyces*, many of which are saprophytes.

### METHODS FOR THE DIAGNOSIS OF DISEASES DUE TO ASPERGILLI AND PENICILLIA

Many species of *Aspergillus* and *Penicillia* are found in Nature, and are among the commonest saprophytic molds. Their spores are practically ubiquitous, and therefore they may contaminate culture media, various uncovered specimens, open wounds, etc.

Many species have been described as pathogenic which are probably accidental, secondary contaminants.

One species of *Aspergillus*, the *Aspergillus fumigatus*, is pathogenic, producing pulmonary aspergillosis.

Collection of specimen, examination of culture, and animal inoculation should be conducted as described under Coccidia, the *Aspergillus* being associated with pulmonary and cutaneous aspergillosis.

These molds are quite complex, are of large size, and are best studied by carefully removing a portion of the medium upon which the mold is growing. This may be placed on a slide and examined with the low power of the microscope.

**Aspergillus.**—There are many species of *Aspergillus*, showing on culture media various colors, and developing a low velvety growth, black, white, yellow, etc. The *Aspergillus fumigatus* produces a brownish colony on neutral or alkaline media, and slightly greenish colony on acid media.

On microscopic examination, the mycelium is septate. Numerous aerial hyphae terminate in an expanded portion from which arise many short conidiophores, at the end of which are a chain of 4 or more round conidia. Ascospores are sometimes seen (See Fig. 215).

**Penicillium.**—The *Penicillium* is much smaller. When fully developed it produces a dirty greenish velvety growth. If a portion is examined under the microscope a septate mycelium will be found with aerial hyphae which branch and rebranch, forming conidiophores which terminate in 6 to 8 spores (See Fig. 215).

**Mucors.**—The *Mucors* are a group of nonpathogenic molds which are likewise commonly met with in contaminations of culture media, etc. They are much larger than the *Aspergilli* and *Penicillia*, and on culture media their hyphae resemble cotton fibers, and the colonies look somewhat like tufts of cotton. From the main mass of the mold, aerial hyphae extend terminating in a black, tiny spore-case visible with the naked eye. Under the microscope it will be seen to be made up of a covering or perithecium containing a large number of small refractile spores. If the mold is examined under a coverglass the trauma will fracture the spore-case, and it will be seen as a collapsed capsule with the spores outside.

# DIAGNOSTIC VIROLOGICAL METHODS

## PRINCIPLES

1. While some of the filterable viruses are successfully cultivated in living tissue cells and especially the chorioallantoic membrane of the developing chick embryo, methods of cultivation are not employed for diagnostic purposes.
2. While intracytoplasmic inclusion bodies are found associated with vaccinia, variola, rabies, trachoma, molluscum contagiosum and certain other virus diseases, examinations for them for diagnostic purposes are conducted only in the case of rabies. Intranuclear inclusion bodies occur in the lesions of yellow fever, herpes, varicella and some other virus diseases, but examinations for them are not ordinarily employed for diagnostic purposes.
3. Animal inoculation tests, however, are of value in the diagnosis of rabies, smallpox, psittacosis and, possibly, in Hodgkin's disease.
4. Biopsy examinations are of value in the diagnosis of lymphopathia venereum and its differential diagnosis from granuloma inguinale, chancroid and carcinoma; likewise in the diagnosis of yellow fever.
5. Serum protection tests are also of value in the detection of susceptible and immune individuals to yellow fever.

## METHODS FOR THE LABORATORY DIAGNOSIS OF RABIES

1. Rabies is a disease of dogs, cats, coyotes, wolves and other animals caused by a filtrable virus occurring in the saliva and transmissible to man and other animals through bites and other injuries.
2. Laboratory diagnosis is made by the examination of smears and sections of the hippocampus major (cornu ammonis) and cerebellum for *Negri bodies*; also by the inoculation of rabbits or guinea-pigs with emulsions of brain substance.
3. When removing the brain from an animal wear heavy rubber gloves; it is also advisable to wear goggles. All instruments should be boiled immediately and the table disinfected by wiping with formalin solution.
4. For removing the brain dissect away the skin and muscles and saw through the head longitudinally, thus separating the two hemispheres.
5. The diagnostic Negri bodies can be found in about 96 per cent of instances by preparing simple touch preparations of the hippocampus major, cerebellum and cerebral cortex (near the rolandic fissure) as follows: Press a scrupulously clean slide, free from flaws, several times against the cut surface with sufficient pressure to cause the tissue to spread out over the slide, leaving thin, even films upon the surface.
6. Place slides in absolute alcohol for 1 to 5 minutes.
7. Remove from alcohol and allow to dry in air.
8. Cover smears with the following stain and steam gently for 2 to 3 minutes:

Sat. alc. sol. fuchsin.....	5 to 8 drops
Löffler's meth. blue .....	15 cc.
Distilled water sufficient to .....	50 cc.

9. This stain will keep fairly well in a refrigerator.



10. Wash with water and dry.

11. Locate the ganglion cells with low power objective and then examine the cells with an oil immersion objective for Negri bodies, which appear as round or oval bodies, variable in size, stained magenta and sometimes showing bluish dots or granules (Plate XI). Nerve cells appear light blue; erythrocytes are salmon or yellow.

12. *Mann's* method is also satisfactory as follows: Without drying, fix the smears with methyl alcohol for 2 or 3 minutes. Stain for 5 minutes with the following:

1 per cent aqueous solution of Grubler's methylene blue . . .	35 cc.
1 per cent aqueous solution of Grubler's B. A. eosin . . . . .	35 cc.
Distilled water . . . . .	100 cc.

Wash in water and rapidly pass the smears through 50, 75, 95 and two changes of absolute alcohol, clear in a mixture of equal parts of xylol and oil of cloves and examine microscopically. When properly prepared and stained, the nerve cells appear light blue and the Negri bodies pink. Erythrocytes stain an orange color.

13. If direct smears are negative prepare sections as follows: (a) Place pieces of tissue not over 1 by 1 by 0.2 centimeter in 10 parts of Zenker's fluid for 8 hours. (b) Wash in running water for 8 to 24 hours. (c) Place in 80 per cent alcohol for 1 hour and then in 95 per cent and absolute alcohols for 1 hour each. (d) Place in xylol for 1 hour and then in xylol-paraffin and finally paraffin in the incubator for 1 hour each. (e) Imbed in paraffin and cut thin sections. (f) Stain by Mallory's or Goodpasture's methods. *Seller's stain* is quite satisfactory:

SOLUTION A

Methylene blue . . . . .	15 gm.
Methyl alcohol . . . . .	100 cc.

SOLUTION B

Basic fuchsin . . . . .	32 gm.
Methyl alcohol . . . . .	100 cc.

Just before it is to be used, mix 150 cc. of solution A with 50 cc. of solution B and 250 cc. of methyl alcohol. This mixture is not stable and should be used the same day it is made. Test the stain before use on brain tissue preferably on tissue containing Negri bodies. The chromatin should stain blue and cytoplasm red. If a clear-cut differentiation of cytoplasm and chromatin is not obtained additional amounts of either solution A or B is added until the desired effect is obtained.

The following method of making the preparations of the brain is suggested by P. T. Buck. Cut a fairly thin section of the brain to be examined and put on the end of a piece of cork stopper, pressing the edges firmly against the cork. Dip a clean slide into 0.4 per cent salt solution and shake off excess. Press the brain tissue on the cork gently against the prepared slide. A thin section of the brain tissue adheres to the slide and a very satisfactory preparation is thus made.

14. If direct smears and sections are negative, rabbit inoculation should be done. If the brain is fresh and uncontaminated, it may be inoculated at once. Otherwise place in pure glycerol and hold in a refrigerator for 2 or 3 days before inoculation.

Emulsify a small piece of hippocampus major in a small amount of sterile saline and inject about 0.1 to 0.2 cc. subdurally in an anesthetized rabbit by forcing the

## PLATE XI



### NERVE CELLS CONTAINING NEGRI BODIES

Hippocampus impression preparation, dog. Van Gieson stain.  $\times 1000$ . 1, Negri bodies; 2, capillary; 3, free red blood corpuscles.

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)



needle through the thin bone lying in the depression just posterior to the eye or the intralingual route of injection may be used.

As a general rule the average street virus will cause death in about 16 days, but sometimes requires 3 weeks or longer (paralysis). It is, however, necessary to keep the animals under observation for 60 to 90 days before reporting negative results.

If death occurs within 6 days, it is probably due to bacterial infection and the test should be repeated with glycerolated tissue.

If the animal dies of rabies Negri bodies may be demonstrated in the brain tissue, but they are usually smaller and more easily distorted than those produced by street virus.

The inoculation test may also be conducted with guinea-pigs and mice. Willett and Sulkin (*Am. Jour. Pub. Health*, 29: 921, 1939) have found mice more susceptible to the virus than guinea-pigs.

### **METHODS FOR THE LABORATORY DIAGNOSIS OF SMALLPOX**

1. A mixed precipitation and agglutination test may be conducted by mixing in a small test tube 0.5 cc. of a suitable suspension in saline solution of material removed from skin lesions with 0.5 cc. of an antivaccinal rabbit serum, followed by incubation in a water bath at 37° C. for an hour. A control should be set up at the same time using normal rabbit serum. A positive reaction is marked by the appearance of a finely floccular precipitate. This test is a practical method for the rapid diagnosis of doubtful cases of smallpox.

2. A second test consists of the intracutaneous inoculation of about 0.1 cc. of a heavy suspension of variolous material in sterile saline solution into a normal rabbit. A red palpable swelling develops by the second day, reaching its maximum about the fourth day when it appears as a firm, elevated lesion about 10 to 20 mm. in diameter. Subsequently the central area becomes straw-colored with desquamation or crust formation followed by healing by the twelfth day with no scar. At the same time it is advisable to inoculate a vaccinated rabbit, in which case either no reaction occurs at all, or there is an allergic reaction characterized by the rapid appearance and disappearance of a red tense nodule. Material from varicella, as a rule, gives rise to no reaction in either normal or vaccinated rabbits, although on this point the evidence is conflicting.

3. Paul's test is conducted by anesthetizing both eyes of a rabbit and gently scarifying the corneae with a fine needle, making 4 or 5 horizontal and as many vertical scratches, about 1 mm. apart. A loopful of a heavy suspension of material in sterile saline solution is then gently rubbed over the scarified area of one eye, the other serving as a control. In about 48 hours a keratitis with a small umbilicated papule develops in the inoculated eye but, according to Defries and McKinnon, positive reactions are observed in only 50 per cent of cases.

### **METHOD FOR THE LABORATORY DIAGNOSIS OF PSITTACOSIS**

The method of Rivers and Berry (*Jour. Exper. Med.*, 61: 205, 1935) is as follows:

1. Material coughed up from the lungs—not saliva or discharges from the nasopharynx—is washed and then emulsified in saline solution or Locke's solution by

means of repeated passages through a 20-gauge needle attached to a syringe. Six white mice are inoculated intraperitoneally with the emulsion, 3 receiving 0.25 cc. each, while 3 others get 0.5 cc. each. Too rich an emulsion should not be used because the mice must be able to destroy the bacteria in order that the virus may be obtained free from contaminants. The animals are observed for a period of 30 days.

2. At times, however, the bacteria in the sputum are so virulent that it is impossible to test for psittacosis virus in their presence. Then it is essential to filter the sputum and inoculate mice with the filtrate in the following manner: The patient's sputum, to which 20 to 50 volumes of meat infusion broth, pH 7.8, and a small amount of alundum have been added, is thoroughly ground in a mortar. The emulsion is centrifuged for 10 minutes at a speed of 3000 r.p.m. Then the supernatant fluid is filtered through a Berkefeld V candle at a pressure of 15 to 30 cm. of mercury. Each of 6 mice receives intraperitoneally, on 3 successive days, 2 cc. of the filtrate. The animals are observed 30 days.

3. The criteria by which the presence of psittacosis in the inoculated mice is determined are as follows:

(a) The development in some or all of the animals of illness which is usually fatal within 5 to 14 days, but occasionally not before 30. If, after the fourth or fifth day, a mouse becomes sick, it should be killed rather than allowed to die.

(b) The absence of ordinary bacterial infections as determined by aerobic and anaerobic cultures from material obtained at necropsy.

(c) The presence in the liver and spleen of the characteristic pathological picture consisting of focal necrotic lesions into and around which there is a collection of polymorphonuclear and mononuclear cells.

(d) The presence of "minute bodies" of psittacosis in impression smears taken from the liver and spleen—particularly the spleen. These bodies stain easily with a modification of Castaneda's methylene blue safranin method:

Phosphate buffer pH 7.0.....	95 cc.
Formalin .....	5 cc.
Löffler's methylene blue.....	10 cc.

Stain 2 minutes with the methylene blue preparation, rinse in tap water, and quickly counterstain with a 10 per cent aqueous safranin solution. The "minute bodies" take a purple or blue stain while the cells are pink.

(e) The establishment of serial passages of the virus in mice by means of liver and spleen emulsions from the animals receiving unfiltered sputum or sputum filtrates.

(f) The demonstration that mice which have lived for 30 days following the inoculations of sputum or sputum filtrates have developed an active immunity against a potent strain of psittacosis virus. The tests for immunity should not be made sooner than 30 days after the primary inoculations, because mice develop an immunity against psittacosis slowly.

All of the above conditions obviously need not be fulfilled in each instance, sometimes one, sometimes another serves to establish a diagnosis.

### **METHODS FOR THE LABORATORY DIAGNOSIS OF LYMPHOPATHIA VENEREUM**

1. Intradermal and complement fixation tests are of most value in the diagnosis and differential diagnosis of lymphopathia venereum (lymphogranuloma venereum); these are described in succeeding chapters.

2. Biopsy examinations, however, are useful and particularly in doubtful cases and to exclude malignancy. In the bubo form there are inflammatory and suppurative changes in the lymph nodes as well as the perilymphoid tissue. There is a polymorpho-granulomatous reaction; tiny foci of necrosis or micro-abscesses surrounded by macrophages with occasional giant cells are seen. In the elephantiasic forms there is a pronounced peritubular infiltration with plasma cells and lymphocytes, accompanied by fibroblastic activity, dilated lymphatics and in some cases with giant cells of the foreign body type. Occasionally, foci of suppuration are noted similar to those seen in buboes.

### **METHODS FOR THE LABORATORY DIAGNOSIS OF HODGKIN'S DISEASE**

1. The laboratory diagnosis of Hodgkin's disease is almost entirely based upon the histological examination of lymphatic glands removed by biopsy.

2. While the etiology of the disease is unknown, it has been suggested by Gordon (*Brit. Med. Jour.*, 1: 641, 1933) that it may be due to a filtrable virus. This is based upon the observation that the intracerebral inoculation of rabbits with suspensions of the lymph nodes produces an encephalitic syndrome in a large percentage of cases. The reaction appears to be due to some other factor because of the absence of intracellular inclusion bodies and the fact that the encephalitis cannot be transmitted from rabbit to rabbit. False positive reactions have been reported following inoculation with tuberculous lymph nodes and various normal tissues although Goldstein (*Am. Jour. Med. Sci.*, 191: 775, 1936) observed positive reactions with the nodes of 7 of 9 cases of Hodgkin's disease with no false positive reactions due to inoculation with the nodes of 20 controls including 9 tuberculous nodes and 2 from cases of infectious mononucleosis. It would appear, therefore, that the Gordon test might serve as a helpful laboratory test for Hodgkin's disease even though the etiology of the disease and the mechanism of the reaction are unknown.

### **METHOD FOR THE LABORATORY DIAGNOSIS OF YELLOW FEVER**

The only laboratory examination of direct value in the diagnosis of yellow fever is the histological examination of tissue removed by viscerotomy. This consists of removing liver tissue postmortem from persons dying less than 11 days after the onset of any febrile illness, regardless of the clinical diagnosis. Because of the characteristic lesions of yellow fever, histological examinations of such specimens enables one to determine with considerable accuracy whether or not the disease was yellow fever. Many thousands of liver specimens have been examined and the presence or absence of yellow fever determined in regions where it would have been otherwise impossible.

**METHOD FOR THE DETECTION OF IMMUNITY IN YELLOW FEVER**

1. The Sawyer and Lloyd serum protection test in mice (*Jour. Exper. Med.*, 54: 533, 1931) is highly sensitive and useful in epidemiological investigations to determine whether individuals have ever had yellow fever, and in tests to find whether vaccinated persons have been successfully immunized. If the serum lacks protective power, the mice die of yellow fever encephalitis.

2. Collect sufficient blood for yielding at least 6 cc. of serum. The test requires 3 cc. The remainder is used in case the results of the first test are doubtful or the control tests prove unsatisfactory. Sera markedly contaminated with bacteria are unsatisfactory.

3. Healthy young adult mice of about 20 gm. are preferred.

4. Five days before conducting the tests a sufficient number of mice are inoculated intracerebrally with yellow fever virus in mouse brain tissue. Subsequently definitely sick animals are killed with chloroform and the brains removed as aseptically as possible. These are mixed, weighed and finely ground up in a sterile mortar with sufficient 10 per cent sterile normal serum in sterile saline solution to make a 10 per cent suspension.

5. On the day of the test, aseptically inject into the center of the brain of each of 6 mice 0.03 cc. of a solution of starch prepared by dissolving 1 gm. of cornstarch in 50 cc. of normal saline solution, heating in a bath of boiling water and autoclaving at 121° C. for 15 minutes.

6. At the same time give each mouse an intraperitoneal injection of 0.6 cc. of virus-serum mixture prepared by mixing in a sterile test tube 3 cc. of serum and 1.5 cc. of brain-virus suspension.

7. Include tests with normal human serum and a known immune serum (human or monkey) in the same manner as controls.

8. Observe the mice daily for at least 14 days. Deaths before the fifth day are in all probability not due to yellow fever virus. Ordinarily, death beyond the tenth day shows a considerable protective action of the serum with almost the same significance of a survival. The results are reported as "protection", "no protection", "inconclusive", or "unsatisfactory". The "unsatisfactory" results include those in which the controls are unsatisfactory and those in which fewer than 4 mice are alive and well 4 days after inoculation.

9. Ordinarily the identification of the probable cause of death of the mice rests on the known source of the virus and the fact that in the controls the known immune yellow fever serum protects the mice and the normal serum does not.

# PARASITOLOGICAL METHODS

## METHODS FOR PARASITOLOGICAL EXAMINATIONS OF THE FECES

**Principles.**—1. So many diseases and carrier states caused by animal parasites are due to infestation of the gastro-intestinal tract that examinations of the feces for worms, ova, larvae, or cysts, are among the most important of parasitological examinations.

2. Not all parasites or their products occurring in the feces, however, are pathogenic. For example, several species of free-living amebae, as well as certain species of free-living flagellates, may occur as accidental contaminants. These are known as *coprozoic parasites* and are of no significance except for the fact that they may be mistaken for pathogenic species. Furthermore, various other objects such as intestinal yeasts and fungi, vegetable, epithelial and squamous cells, leukocytes and especially large endothelial macrophages which have ingested erythrocytes, as well as such extraneous materials as air bubbles, partially saponified fat globules, starch granules and other foods, and even mucus, may be mistaken for intestinal protozoa, cysts or helminthic ova (Fig. 241) by inexperienced workers.

3. In the routine examination of feces for animal parasites, the following order should be adhered to: (a) microscopic examinations for the trophozoites and cysts of intestinal protozoa, particularly amebae; (b) microscopic examinations for the ova or larvae of the helminthes (worms) and (c) gross examinations of entire specimens of feces for adult parasites or their segments.

### METHODS OF EXAMINATION FOR AMEBAE

Of the Sarcodina, only *Endamoeba histolytica* is definitely pathogenic, producing acute or chronic dysentery, amebic enteritis, abscesses in the liver, lungs or elsewhere and the carrier state (contact or convalescent). *Endamoeba gingivalis* is probably non-pathogenic, but may favor secondary bacterial or spirochetal infections in gingivitis. *Endamoeba coli*, *Endolimax nana*, *Iodamoeba bütschlii* and *Dientamoeba fragilis* are apparently nonpathogenic and harmless commensals, but are important from the standpoint of being possibly mistaken for *E. histolytica* in the laboratory.

**Collection.**—Feces, collected in the usual manner, may be used in examinations for cysts, but when examinations for trophozoites are indicated, the method of collection may be as follows:

1. The specimen should be collected directly in clean, covered receptacles (bed pans, swabs in test tubes containing 0.5 cc. of warm saline solution, syringes, bottles or droppers) preferably sterilized by heat. These receptacles should not be sterilized by chemical disinfectants as protozoa in the vegetative stage are easily killed and quickly undergo autolysis in the presence of small amounts of such chemical agents.



## METHODS FOR EXAMINATION OF THE FECES

uticles are not properly cleaned and sterilized, there is always the possibility of introducing free-living protozoa into the specimen and thus confusing the findings. One should be cautioned not to pass urine with the feces.

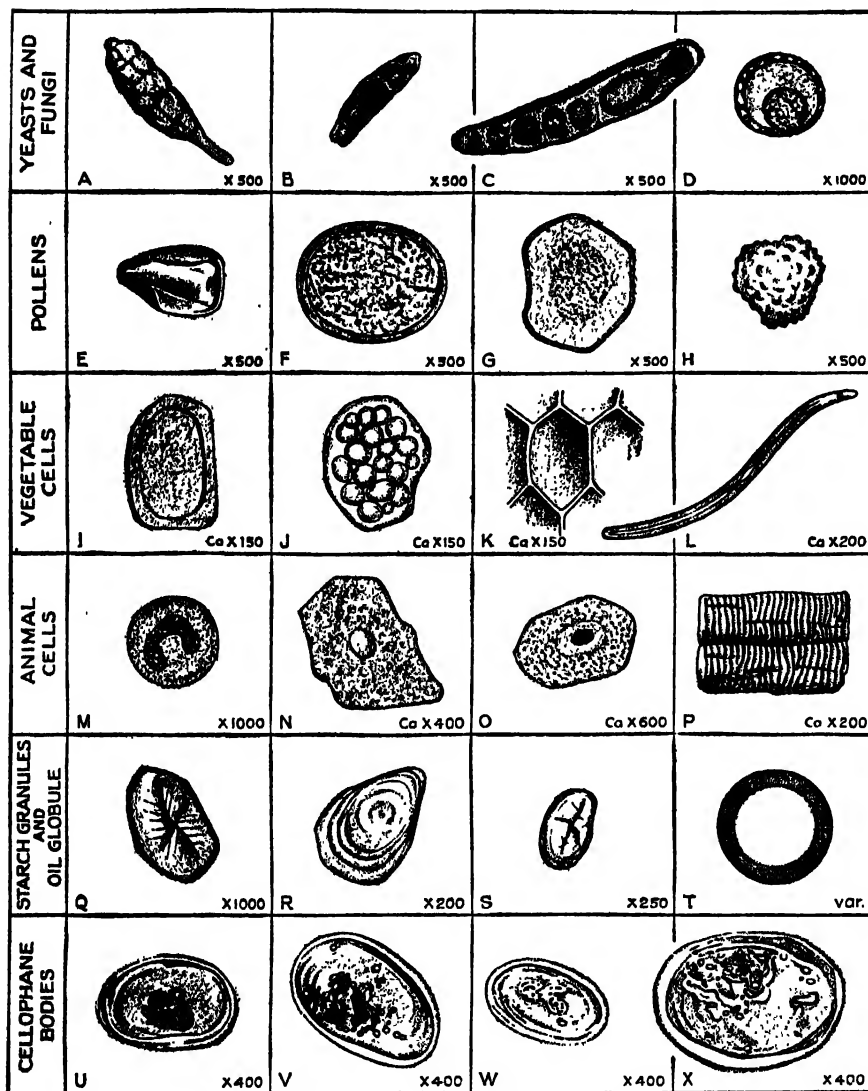


FIG. 241.—OBJECTS IN FECES SOMETIMES MISTAKEN FOR PROTOZOAN CYSTS AND HELMINTHIC OVA

A, *Alternaria* spore; B, *Acrothecium* spore; C, *Helminthosporium* spore; D, *Blastocystis hominis*; E, hemp pollen; F, orchard grass pollen; G, timothy pollen; H, ragweed pollen; I to L, vegetable cells; M, leukocyte; N, squamous epithelial cell; O, epithelial cell; P, muscle fiber; Q, cornstarch; R, potato starch; S, rice starch; T, oil globule; U to X, cellophane bodies, (redrawn from Reardon, 1938). (From Belding, *Textbook of Clinical Parasitology*, D. Appleton-Century Co., New York.)

2. Specimens should be kept in the original receptacles used for collection until examined. All specimens should be examined as soon as possible after collection since

protozoa degenerate rapidly and the possibility of an accurate diagnosis diminishes as the time between collection and examination of the specimen increases. *If a delay in examination occurs the material should be kept at or near 37° C.* as all protozoa are quite sensitive to chilling and are rapidly killed by temperatures of 45° C. or higher. Since drying also affects them, the specimen should have its original moisture when presented for examination.

3. If the lesions are in the rectum or sigmoid, specimens may be obtained by means of the proctoscope or sigmoidoscope. These are more likely to yield protozoa than feces passed in regular way. However, because of the attending discomfort and probable pain to the patient, these methods should be used only after it has been demonstrated that the passed feces are negative.

4. It is practically impossible to find protozoa in a stool after an oil cathartic or following a barium meal. Specimens collected by means of an enema are also unsatisfactory. Therefore, if any of the above have been used, examinations for protozoa in the feces should be delayed for at least 72 hours.

**Method for Trophozoites.**—1. If the stool is fluid, select blood stained portions of mucus or minute tissue fragments for examination. If semisolid and free from blood or mucus, any part may be used. In formed stools, it is best to make preparations from the surface as the trophozoites or motile amebae are most apt to be found in this location.

2. Warm a clean slide so that it feels comfortable when touched to the back of the hand. Then secure a small amount of mucus, or mucus and blood, by means of a wire loop or wooden applicator and thoroughly emulsify it in 1 drop of warm normal saline solution on the middle of the slide. It is advisable to prepare a second slide at the same time, using distilled water, as recommended by Hakansson, for the purpose of aiding in differentiating the trophozoites of *E. histolytica* from those of *Dientamoeba fragilis* and *Endolimax nana*. A convenient method of keeping a slide warm is to place a heated penny on one end.

3. Take a clean No. 1 coverglass between the thumb and forefinger of the right hand, contact the slide with one edge of the coverglass near the drop, but not touching it, push the coverglass along the surface of the slide until its edge contacts the drop, rock it slightly from side to side to allow a portion of the fluid to come under the edge of the coverglass, and then let the coverglass drop from between the fingers, allowing it to fall on the slide. The fluid portion of the drop on the slide will then automatically be drawn by capillary attraction under the coverglass, while the solid particles will be excluded. This method insures a thin, even preparation of not too great density and insures even apposition of the coverglass to the slide. The preparation is now ready for examination, but if it is to be kept on a warm stage for any period of time, it should be ringed with vaselin.

4. Examine with the low and high power objectives, shutting down the light considerably.

5. The trophozoites of *E. histolytica* (Fig. 242) must be differentiated from those of *E. coli* and *Endolimax nana*. For this purpose see Table 25. Differentiation is based upon (1) size and color; (2) differentiation of ectoplasm and endoplasm; (3) granularity of endoplasm and presence of cell inclusions; (4) the visibility of the nucleus, location when in motion, and size; (5) type of motility (active, sluggish, progressive or nonprogressive); (6) pseudopodia (single or multiple, clear or granular); (7) flow-

ing of endoplasm into pseudopod (slow or explosive) and (8) presence or absence of erythrocytes or bacteria in the endoplasm. *Never make a diagnosis of the trophozoits of amebae unless they are motile.*



FIG. 242.—ENDAMOEBA HISTOLYTICA

Motile forms showing ingested red cells and clear ectoplasm. (After Army Medical School Collection, Washington, D. C.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, D. Appleton-Century Co, New York.)

6. According to Hakansson, in preparations with normal saline solution the trophozoites of *Dientamoeba fragilis* at rest present a faultlessly circular outline and, if motile, characteristic thin veil-line pseudopodia with sharp points and corners. In preparations with water the ectoplasm of these trophozoites swells and ruptures in 1 to 10 minutes with extrusion of its contents. The trophozoites of *E. nana* and *I. bütschlii* distend and disappear without explosive rupture or evacuation of contents while those of *E. histolytica* and *E. coli* may rupture with extrusion of the endoplasm and nucleus, but never with complete emptying and restoration of the ectoplasmic shell as in *D. fragilis*.

7. Tissue cells derived from the patient, or ingested food, may at first glance appear as amebae, but careful examination of them will easily establish their true nature. Macrophages may be found containing phagocytized erythrocytes, but examination reveals their typical nuclear structure and ameboid movement is not observed. Epithelial cells are pale in color and have nuclear characteristics which easily differentiate them. Vegetable cells, such as starch granules, pollen granules, yeast cells, or other cells of this type, have a certain definiteness of outline and structure that should lead to no confusion (Fig. 241).

**Methods for Cysts.**—1. On a slide thoroughly emulsify a loopful of feces in normal saline solution. A second slide should be prepared at the same time using water for the purpose of aiding in distinguishing cysts of *E. histolytica* from *Blastocystis hominis*. According to Hakansson blastocysts, being subject to osmosis, distend, rupture, and rapidly disappear, which greatly aids in detecting the amebic cysts.

2. Add a drop of iodine solution (2 gms. iodine, 4 gms. potassium iodide and 100 cc. distilled water) or a drop of iodine-eosin solution (Donaldson). This is prepared by mixing 2 cc. of a saturated solution of eosin in normal saline solution, 1 cc. of iodine solution (100 cc. saline solution, 5 gms. potassium iodide and iodine crystals to saturation) with 2 cc. of saline solution.

**TABLE 25**  
**DIAGNOSTIC POINTS IN THE DIFFERENTIATION OF ENDAMOEBA**  
**HISTOLYTICA, ENDAMOEBA COLI, AND ENDOLIMAX NANA \***

	Endamoeba histolytica	Endamoeba coli	Endolimax nana
<b>Vegetative or Trophozoite stage. Unstained.</b>			
Size	18 to 60 micra; average, 20 to 25 micra	15 to 50 micra; average, 20 to 30 micra	6 to 12 micra, average 8 micra
Motility	Actively progressive and directional	Sluggish; rarely progressive; not directional	Sluggishly progressive
Pseudopodia	Finger-shaped, clear and glass-like	Shorter and more blunt; less glass-like in appearance	Broad and blunt; not glass-like
Inclusions	Red blood corpuscles when feces contains blood; no bacteria in fresh specimens	Numerous bacteria, crystals, and other materials; no red blood corpuscles	Numerous bacteria; no red blood corpuscles
Nucleus	Invisible	Visible	Visible
<b>Vegetative or Trophozoite stage. Stained.</b>			
Nuclear membrane	Delicate; inner surface has single layer of minute chromatin dots	Thicker; inner surface lined with coarse chromatin dots	Intermediate in thickness; chromatin rarely seen on inner surface
Karyosome	Very small. Usually in center of nucleus	Twice as large, situated eccentrically	Large and may be divided into one large and one small mass, situated at one side or in center of nucleus
Intranuclear chromatin	No chromatin between karyosome and membrane	Chromatin grains between karyosome and nuclear membrane	No chromatin between karyosome and membrane
Inclusions	Red blood corpuscles; no bacteria in fresh specimens	No red blood corpuscles; many bacteria and other material	No red blood corpuscles; many bacteria
<b>Cystic Stage of Development. Iodine stain</b>			
Size	6 to 20 micra; average 7 to 15 micra	10 to 20 micra; average 12 to 18 micra	5 to 10 micra
Shape	Generally spherical; may be oval and rarely irregular	Spherical; rarely oval or irregular	Spherical, oval or ellipsoidal
Nucleus	One to four; minute karyosome in center	One to eight; eccentric karyosome	One to four; large karyosome central or to one side
<b>Hematoxylin Stained Cysts</b>			
Size	As in iodine-stained specimens	As in iodine-stained specimens	As in iodine-stained specimens
Nuclear structure	Delicate membrane, minute central karyosome, no chromatin between karyosome and membrane, minute grains on nuclear membrane	Thicker membrane, larger eccentrically located karyosome, chromatin grains between nuclear membrane and karyosome, and large granules on nuclear membrane	Thick nuclear membrane, large central or divided karyosome
Chromatoidal bodies	Bar, oval or thick rod-like masses; present in about 50 per cent of the cysts	Filamentous or spicular with square or pointed ends; present in less than 10 percent of cysts	Small granular or bacilliform masses, not comparable with those seen in the other species
Nuclei, number of	One to four	One to eight	One to four

\* From Charles F. Craig, *Amebiasis and Amebic Dysentery*, Charles C. Thomas Co.

*D'Antoni's standardized iodine solution* is made from a standardized potassium iodide solution which is adjusted, by the specific gravity method, to an exact 10 per cent strength. To 100 cc. of a 1 per cent solution 1.5 gm. of powdered iodine crystals are added and, after standing 4 days, this stock solution is ready for use. It may be kept for long periods, if tightly stoppered. For immediate use, a small portion is filtered into a dropping bottle (should be discarded after 10 days). Sealed tubes of potassium iodide (10.0434 gm.) and glass-stoppered bottles of iodine (15 gm.) to make 1000 cc. of stock solution may be purchased.

3. Mix thoroughly and cover with a coverglass. The thickness of these preparations should be such that a typed page can be read easily through them.

4. Examine under the low and high power.

5. With the iodine solution, the protoplasm of cysts appear a lemon yellow while the nuclear membrane and karyosomes are brighter and refractile (Fig. 243). Glycogen assumes a dark brown color.

6. With the iodine-eosin solution, the cysts take on a yellow or brown color and stand out clearly. The nucleus also becomes clearly defined. Bacteria, yeasts and fecal particles stain pink.

7. If cysts are not found in these ordinary preparations the *concentration method of Craig* may be employed as follows: (1) Emulsify a portion of stool about the size of a pea in 10 cc. of water or saline solution; (2) strain the emulsion into a centrifuge tube through 2 layers of cheesecloth, add sufficient saline solution to fill the tube and mix thoroughly; (3) centrifuge at moderate speed for 5 minutes; (4) secure sediment with a wide-mouthed pipet; (5) prepare unstained and stained preparations and examine for cysts.

The *brine flotation method* is quite satisfactory as follows: (1) Prepare a fecal suspension by emulsifying 1 part stool (about the size of a pecan) in 10 parts lukewarm water; (2) strain 10 cc. of this emulsion through 2 layers of wet cheesecloth into a small test tube or centrifuge tube; (3) centrifuge for 45 to 60 seconds at approximately 2500 r.p.m., pour off the supernatant fluid, and add 2 or 3 cc. of water.

FIG. 243.—PROTOZOAN CYSTS STAINED WITH IODINE STAIN

FIG. 243.—Nos. 12, 13, 14 and 15 and *Entamoeba histolytica*. Note nuclei with central karyosomes, chromatoidal masses in 12, 13 and 14, and dark glycogen masses in 12 and 13. Medium size cysts are represented in 12, 13 and 14, and small size cysts in 15.

Nos. 16, 17 and 18, *Endamoeba coli*. Note nuclei with eccentrically situated karyosomes, and thicker nuclear membrane, and absence of the large chromatoidal masses present in the cysts of *Entamoeba histolytica*.

Nos. 19 and 20, *Endolimax nana*. Two and four nucleated cysts.

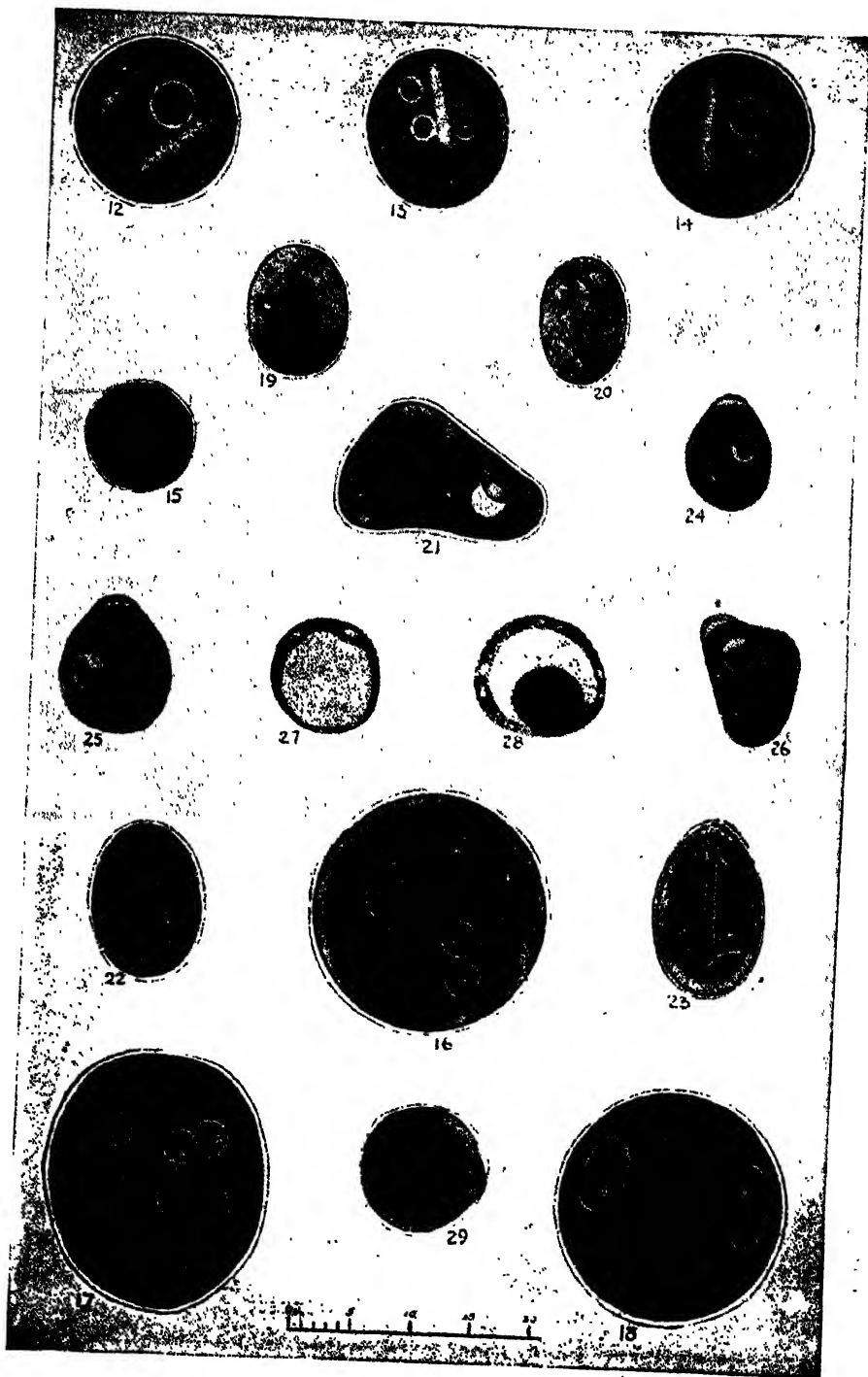
No. 21, *Iodamoeba buitschlii*. Note large glycogen vacuole and nucleus with indistinct membrane and large, refractive, eccentric karyosome.

Nos. 22 and 23, *Giardia lamblia*. Note shape of cysts and presence of axostyles and parabasal bodies as refractive lines. Nuclei are four in number and minute.

Nos. 24, 25 and 26, *Chilomastix mesnili*. Note lemon-shaped cysts containing a large nucleus with eccentrically situated karyosome and refractive fibrils representing cystostomal structures.

Nos. 27 and 28, *Blastocystis hominis*. Note nuclei situated in the outer wall which encloses a large vacuole which may contain inclusions.

No. 29, *Phycomycete*. Note absence of nuclei. These spores are frequently mistaken for cysts of *Entamoeba histolytica* but the absence of nuclei or chromatoidal bodies should serve to distinguish them. All figures are  $\times 1675$ . (From Boeck and Stiles, *Hyg. Lab. Bull.*, No.



Then break up the sediment and repeat the above, centrifuge and discard the supernatant fluid 3 or 4 times; (4) after pouring off the last supernatant fluid, add 3 to 4 cc. of 33 per cent aqueous solution of zinc sulfate, break up the packed sediment and add enough zinc sulfate solution to fill the tube about  $\frac{1}{2}$  inch from the rim; (5) centrifuge the tube for 45 to 60 seconds at top speed; (6) remove several loopfuls of the material floating on the top surface film to a clean slide. Add 1 drop of iodine solution or iodine-eosin solution and coverglass.

8. *Mayer's haemalum stain method* is quite satisfactory for the preservation and differentiation of cysts; the technic is as follows:

(a) Quickly smear suitable portions of the material on slides or coverglasses, and at once immerse in Schaudinn's sublimate alcohol fixing solution, previously warmed to body temperature (37° C.):

Saturated bichloride of mercury..... 2 volumes  
Absolute alcohol (or 96 per cent alcohol).... 1 volume

Mix the alcohol and saturated solution of bichloride of mercury, and add 5 per cent glacial acetic acid immediately before using.

If the material floats off the slide, it will be necessary to lightly coat the slide or coverglass first with egg albumin or serum before the material is spread upon it. Thick preparations should be avoided. Several smears should be prepared, since some will stain poorly.

(b) Let remain in fixing solution 10 to 20 minutes (10 minutes for vegetative forms, 20 minutes for cysts).

(c) For a few minutes (5), rinse the preparation with 50 per cent alcohol to remove the sublimate.

(d) Rinse for a few minutes (5) in 70 per cent alcohol with enough iodine to give a rich port wine color.

(e) Dehydrate by successively passing through 50, 70, and 96 per cent alcohol. Preparations may remain in 96 per cent alcohol until it is convenient to stain them.

(f) Carry the preparations successively through 70, 50 and 30 per cent alcohol, allowing them to remain 5 minutes in each, and then place in distilled water.

(g) Immerse in the Mayer's haemalum stain from 5 to 20 minutes (5 to 10 minutes for vegetative forms, full time for cysts):

Hematoxylin crystals ..... 1 gm.  
Sodium iodate ..... .2 gm.  
Potassium alum ..... 50 gm.  
Distilled water ..... 1000 cc.

The hematoxylin is dissolved in the distilled water, and the sodium iodate and potassium alum are then added and dissolved. The mixture is then ready for use. It should be a deep red color. When it turns brown, and when a precipitate forms, it is no longer usable.

(h) Wash gently in running tap water until they appear blue in color.

(i) Dehydrate by carrying the preparations successively through 30, 50, 70, 90 and absolute alcohol, allowing them to remain in each 5 minutes.

(j) Place in equal parts of absolute alcohol and xylol for 5 minutes.

(k) Clear in xylol.

(l) Mount in xylol balsam.

(m) At no time must the smears be allowed to dry during the entire procedure.

9. *Heidenham's iron-hematoxylin* method, although more time-consuming than Mayer's method, gives much better differentiation. Several slides should be stained at a time, since only a few will give good results. The technic is as follows:

(a) Prepare film by smearing a small amount of material on a clean coverglass so as to form a thin, moist film.

(b) Without allowing the preparation to dry, fix from 10 to 20 minutes in Schaudinn's fluid by floating the coverglass, film side down, on the solution which has previously been warmed (about 37° C.). The fluid may be made up in sufficient amount to fill the lower half of a Petri dish. Following the period of fixation, the preparation should be turned over so that the film side is up.

(c) Drain off the fixative and cover with 50 per cent alcohol, agitating the dish. Repeat this process several times to remove all traces of fixative. Drain and

Cover with 70 per cent iodine-alcohol for 3 to 5 minutes. Drain and

Cover with 70 per cent alcohol 3 to 5 minutes. Drain and

Cover with 85 per cent alcohol 3 to 5 minutes. Drain and

Cover with 95 per cent alcohol 3 to 5 minutes. Drain and

Cover with 85 per cent alcohol 3 to 5 minutes. Drain and

Cover with 70 per cent alcohol 3 to 5 minutes. Drain and

Cover with 50 per cent alcohol 3 to 5 minutes. Drain and

Cover with 30 per cent alcohol 3 to 5 minutes. Drain and

Wash in several changes of tap water for 10 to 20 minutes. Drain and

(d) Cover with mordant for 15 minutes in incubator at 37° C. *Note:* Better preparations may be obtained by allowing the mordant to act over night in the incubator. (If pressed for time, *steam* gently for 15 minutes):

#### THE MORDANT

Ferric ammonium sulphate ..... 4 gms.

Distilled water ..... 100 cc.

(e) Drain off the mordant and wash in several changes of tap water 5 to 10 minutes.

(f) Drain and place in 0.5 per cent aqueous solution of hematoxylin for the same period as used in the mordant stage:

Hematoxylin (any standard hematoxylin) .. 0.5 gms.

Distilled water ..... 100 cc.

Dissolve the hematoxylin in a small amount of 95 per cent alcohol (about 5 cc.). Dilute to 100 cc. with distilled water. Let ripen for at least 2 days before using.

(g) Differentiate in fresh 2 per cent aqueous solution of ferric ammonium sulphate (iron alum). *Note:* This is the most critical step in the whole procedure and must be done carefully by removing the preparation with forceps, dipping into the differentiating solution and quickly rinsing off in a beaker of tap water. The preparation should now be examined under the 4 mm. objective of the microscope to see whether sufficient differentiation has been accomplished. If not, the process is repeated until



the nucleus stands out as a blue-black, with the cytoplasm and debris of a gray or bluish-gray shade.

(*h*) After differentiation, wash in several changes of tap water or running water from 20 to 30 minutes.

(*i*) Dehydrate in graded alcohols: 30 per cent, 50 per cent, 70 per cent, 70 per cent with iodine, 85 per cent, 95 per cent and absolute.

(*j*) Clear in xylol (oil of thyme or oil of cloves may be used), 5 minutes.

(*k*) Mount in balsam.

(*l*) At no time, during the entire procedure, must the smears be allowed to dry.

10. *Sheridan's cellosolve method* (*Jour. Lab. and Clin. Med.*, 27: 254, 1941) saves considerable time and is much less expensive. Cellosolve is ethylene glycol mono-ethyl ether. The method is as follows: (*a*) Fix in Schaudinn's solution for 5 minutes; (*b*) 95 per cent alcohol with iodine to a port wine color for 3 minutes; (*c*) 70 per cent alcohol for 3 minutes; (*d*) rinse in tap water; (*e*) 4 per cent solution of ferric ammonium sulfate for 15 minutes; (*f*) rinse in tap water; (*g*) 0.5 per cent aqueous solution of hematoxylin for 10 minutes; (*h*) 0.25 per cent solution of ferric ammonium sulfate for about 12 minutes; (*i*) wash in running water for 1 minute; (*j*) cellosolve for 10 minutes; (*k*) mount in balsam.

11. The *Markley, Culbertson and Giordano method* (*Am. Jour. Clin. Path.* 13: No. 1, 1943) eliminates the uncertain and troublesome step of destaining. The method is as follows: (*a*) Fix in Schaudinn's solution for at least 2 minutes; (*b*) place in 95 per cent alcohol for 10 to 15 seconds (if permanent mounts are desired, it is advisable to remove mercuric chloride with iodine-alcohol); (*c*) rinse in water; (*d*) apply mordant (5 per cent aqueous solution of iron ammonium sulfate) at 56° C. for 2 to 3 minutes; (*e*) rinse in water; (*f*) stain in hematoxylin solution (0.4 cc. of 10 per cent alcoholic solution of hematoxylin, 0.8 cc. glacial acetic acid and 40 cc. distilled water) for 1 to 2 minutes at 56° C.; (*g*) rinse in water and allow to stand until blue-black (for permanent mounts slides should be washed in running water for 15 to 30 minutes); (*h*) dehydrate in 95 per cent alcohol for 30 to 60 seconds, followed by absolute alcohol or acetone for 60 seconds; (*i*) clear in xylol and (*j*) mount in clarite, Canada balsam, or gum damar. At no time, during the entire procedure, must the smears be allowed to dry.

12. With these methods the cysts of *E. histolytica*, *E. coli* and *Endolimax nana* may be differentiated and identified as shown in Table 25 (Fig. 244). Trophozoites, if present, are likewise stained and differentiated as shown in Table 25 (Fig. 245).

**Cultural Method.**—1. Boeck and Drbohlav's Locke-egg-serum medium (L.E.S.) is generally employed. For composition and method of preparation, see pages 361 to 362.

2. If the stool is solid or semi-solid, select, preferably with aseptic precautions, a portion of feces about the size of a pea; or if fluid, the specimen may be obtained with a wide-mouth pipet fitted with a rubber bulb.

3. Inoculate the L. E. S. medium, taking care to emulsify the feces thoroughly in the fluid portion thereof by rubbing the specimen against the sides of the test tube with a sterile applicator stick or inoculating needle.

4. Place in the incubator at 37° C. for 24 hours.

5. Remove the sediment at the bottom of the fluid portion of the medium with a large-mouth pipet. At least 0.1 of a cc. should be examined.

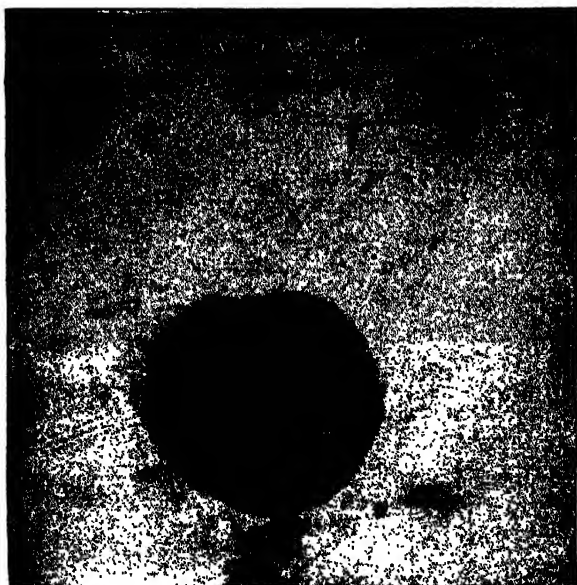


FIG. 244.—ENDAMOEBA COLI CYST SHOWING A LARGE VACUOLE  
(After Army Medical School Collection, Washington, D. C.)

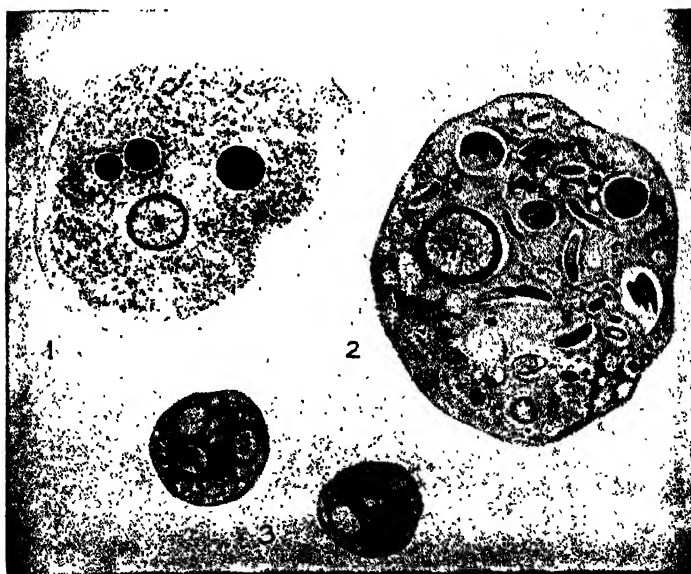


FIG. 245.—THE THREE COMMON INTESTINAL AMOEBAE STAINED WITH IRON HEMATOXYLIN  
1. *Endamoeba histolytica* with three red blood cells; 2, *Endamoeba coli*, and 3, *Endolimax nana* ( $\times 2000$ ). (After Dobell and O'Connor.)

6. Place the sediment on a warm slide, cover with a coverglass, and examine for vegetative forms.

7. A mechanical stage should be used, and every part of the preparation examined as amebae grow quite slowly, and must be searched for thoroughly.

8. If negative results are obtained, place the tube in the incubator for a second period of 24 hour incubation, at which time a second examination should be made. Before considering a culture negative, 3 or 4 preparations should be examined.

Cleveland and Collier's medium (see page 362) is preferred by many for the cultivation of *E. histolytica*.

### METHODS OF EXAMINATION FOR INTESTINAL FLAGELLATES

A *direct* examination of the feces for flagellates may be conducted in the same manner as described for the detection of amebae. *Cultural* methods may be employed, as all intestinal flagellates will survive or grow on the Locke-egg serum (Boeck-Drbohlav) medium with the exception of *Giardia intestinalis*, which has not been successfully cultivated on any medium.

The intestinal flagellates most commonly encountered in the examination of the feces are: (1) *Trichomonas hominis*, (2) *Chilomastix mesnili*, (3) *Giardia intestinalis* (*Lambliia intestinalis*), (4) *Embadomonas intestinalis*, (5) *Enteromonas hominis* (*Trichomonas intestinalis*).

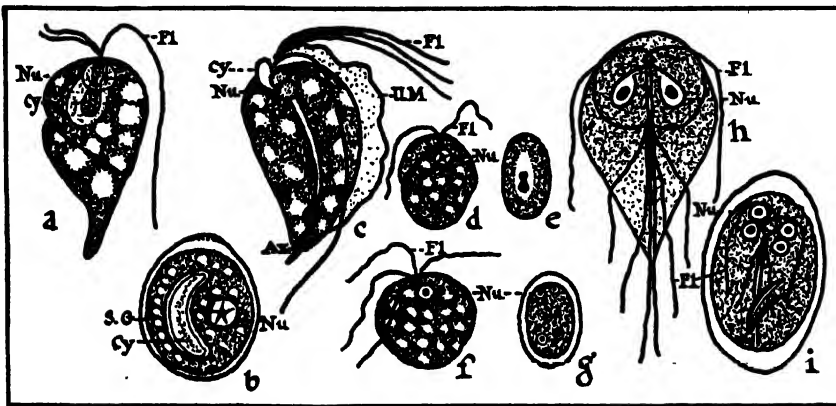


FIG. 246.—INTESTINAL FLAGELLATES (×2500)

a, *Chilomastix mesnili*; b, *Chilomastix mesnili* cyst; c, *Trichomonas hominis*; d, *Embadomonas intestinalis*; e, *Embadomonas intestinalis* cyst; f, *Enteromonas hominis*; g, *Enteromonas hominis* cyst; h, *Giardia intestinalis*; i, *Giardia intestinalis* cyst. (From Belding, *Manual of Human Parasitology*, copyright D. Appleton-Century Co., New York.)

The life cycle of the intestinal flagellates consists of a vegetative or motile form, and a cyst stage. The vegetative form rapidly disappears after leaving the body, but the mature cyst forms are more resistant and may survive for some time. These parasites are considered by most authorities as non-pathogenic, and are found incidentally in the routine examination of the feces.

The cysts are complex in structure. They are approximately the same size as the cysts of amebae, and care must be taken not to confuse them.

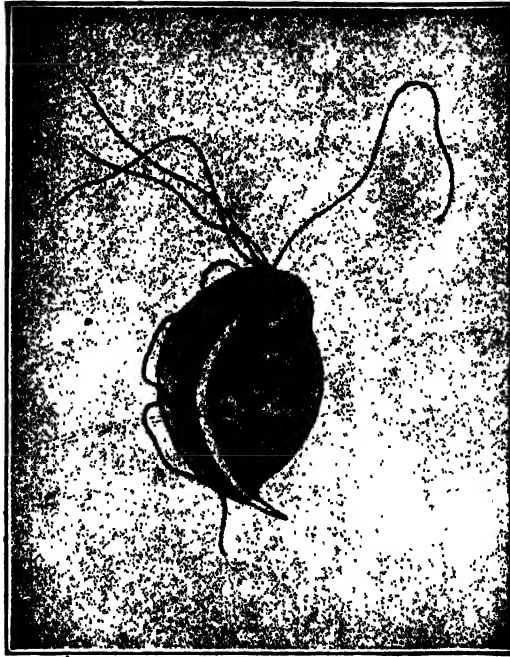


FIG. 247.—TRICHOMONAS HOMINIS

(After Dobell and O'Connor.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

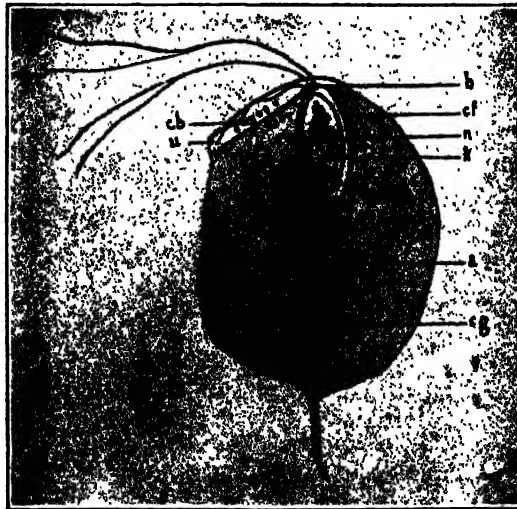


FIG. 248.—TRICHOMONAS VAGINALIS

Camera lucida drawing of a typical specimen  $\times 2250$ , *a*, axostyle, *b*, blepharoplastic granules. *cb*, chromatic basal rod. *cf*, cytostomal fiber. *cg*, chromatic granule. *k*, karyosome. *n*, nucleus. *u*, undulating membrane. (Redrawn from Hegner.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

A schematic representation of the important characteristics of each is shown in Figure 246.

A brief description of each is as follows:

1. *Trichomonas Hominis*.—This flagellate is probably the most common found in the intestine of man (Fig. 247). It measures 9 to 15 micra. Its habitat is the large intestine, and is found in the feces, particularly during diarrhea. It may also occur in the vagina producing vaginitis. It is pear-shaped, and presents four or more flagella, three or more projecting from the anterior end, taking their origin near an oval vesicular nucleus. There is an axostyle extending from the anterior end to the posterior end projecting as a caudal process. An undulating membrane extends along the side and is edged by a flagellary process, which continues posteriorly. A small cytostome is present but may be obscured by structures in the anterior end.

The cyst of this parasite has never been demonstrated. The organism is easily recognized in the feces with low power objective, and is closely related to or identical with the trichomonas found in the vagina (*Trichomonas vaginalis*) and in the mouth (*Trichomonas elongata*). In motion the organism is said to have a cog-wheel appearance. (See Fig. 248).

2. *Chilomastix Mesnili*.—This parasite is probably the next most commonly found in the intestine of man (Fig. 246). It measures 9 to 15 micra. It is sometimes confused with the *Trichomonas hominis* because of its similar shape. The parasite is pyriform with a tapering spine-like process in the posterior end. There is a large cytostome extending about  $\frac{1}{3}$  of the length of the body anteriorly. Four flagella are present, three projecting anteriorly, and the fourth, which is longer, extending posteriorly. The organism inhabits the large intestine, and is occasionally found in the stool.

The cysts are oval in shape,  $8\frac{1}{2}$  micra in diameter. There is a large central nucleus and a laterally placed cytostome. Sometimes degenerate flagella may be seen.

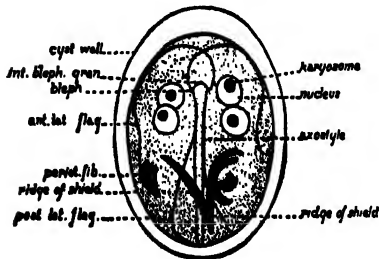


FIG. 249.—CYST OF *GIARDIA INTESTINALIS*

(After Simon.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

3. *Giardia Intestinalis* (*Lambia intestinalis*).—This flagellate is pear or tennis racket in shape, measuring 10 x 20 micra. The dorsal surface is arched. The ventral surface forms a shallow concavity which acts as a sucking disk, and enables the parasite to adhere to the intestinal wall. The structures are paired and symmetrical. Two nuclei are located near the blunt end. Four paired flagella take their origin near this point, giving the parasite a quite characteristic appearance.

The flagellate is very active and exhibits a tumbling movement. Its usual habitat is the duodenum and its presence is often detected by biliary drainage.

The vegetative forms are not usually found except in diarrhea or where a purgative has been given. Cysts, however, may be found in formed feces. The cysts are oval, measuring 8 by 14 micra (Fig. 249).

4. *Embadomonas Intestinalis*.—This organism measures 5 to 6 micra. It is oval shaped and is only occasionally found in the feces of man (Fig. 246). Its habitat is the cecum. It usually has two flagella, one long and slender, the second short and

thick. Nucleus and small cytostome are located at the anterior end. Cysts are small, measuring 4 to 6 micra, slightly pear-shaped, with a single nucleus usually centrally located.

5. *Enteromonas Hominis* (*Tricercomonas intestinalis*).—This is another rare variety of small size, 4 to 8 micra, oval in shape, having a cytostome at the anterior end, 4 flagella and a nucleus. The cysts are tiny, 6 to 8 micra, oval and usually contain 4 nuclei (Fig. 246).

6. *Trichomonas Fetus*.—This flagellate may be present in the uterine or vaginal exudates of cows, the prepuce of bulls, the mucus along the tongue or in the contents of the fourth stomach. It has a spindle shaped body, 10 to 15 micra in length, with 3 anterior flagella and a nearly full-length undulating membrane which continues posteriorly as a trailing flagellum (Wenrich and Emmerson, *Jour. Morph.*, 55: 193, 1933). The method of examination may be as follows: (a) Collect a liberal amount of uterine exudate by means of a catheter (at least 4 ounces), which should be kept in tightly stoppered container during transportation to laboratory (in the case of the bull, swabbings may be wiped carefully about the deepest part of the preputial sac around the base of the glans penis); (b) allow the exudate to stand a few hours to allow formed elements to settle to the bottom; (c) place a drop of fluid from upper layer and a drop of the sediment on another slide and cover with coverglasses (if the material is too thick it may be diluted with saline solution); (d) examine with low power objective and after flagellates are located use high power objective for more detailed study.

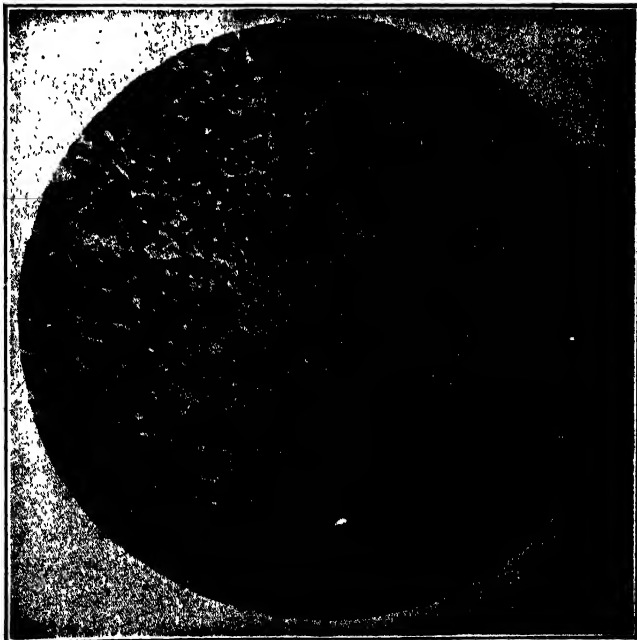


FIG. 250.—BALANTIDIUM COLI

Several balantidia deep in tissue of colon associated with infiltration of lymphocytes. (After Strong.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

### METHOD OF EXAMINATION FOR *BALANTIDIUM COLI*

A number of infusoria have been found in the intestine of man. Only one, *Balantidium coli*, is truly parasitic and of importance. It infests the colon and produces a symptom-complex quite similar to amebic dysentery. The infestation is common among hogs but comparatively uncommon in man, infestation being due to the ingestion of cysts from the feces of hogs. The collection and examination of specimens are similar to those employed for *E. histolytica*. Diagnosis is easy, due to the large size and the active motility of the parasite.

*Balantidium coli* is the largest protozoan parasite of man. It measures 60 to 100 micra in length and 50 to 70 micra in width. It is oval in shape, slightly more pointed at the anterior end. It is covered with fine cilia. At the anterior end is a funnel-shaped mouth, also lined with cilia. Near the middle of the body a large, kidney-shaped macronucleus is found with a small micronucleus adjacent to the concave side. It has a number of vacuoles and a granular cytoplasm (Fig. 250).

The cysts are round, 50 to 60 micra in diameter, having a thick wall, a kidney-shaped macronucleus and a granular cytoplasm.

### METHODS OF EXAMINATION FOR INTESTINAL SPOROZOA (COCCIDIA)

The laboratory diagnosis is made by finding the oocysts in the feces. The collection of specimen and technic of examination are similar to that employed under direct examination of the feces for amebae.

Several species of coccidia have been described as occurring in the feces of man.



FIG. 251.—BLASTOCYSTIS HOMINIS

A peculiar structure related to the yeasts found in feces; stained;  $\times 1000$ . (After Lynch, from Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

It is probable that, with the exception of *Isoospora hominis* and *Isoospora belli*, they are coprozoic and not true parasites. The coccidia are tissue parasites, their rather complex life cycle taking place within the cells of the intestine. The oocysts are found in the feces.

The *Isoospora belli* oocysts measure approximately 28 by 14 micra, have a colorless wall, are somewhat pear-shaped, and when passed in the feces, are unsegmented, containing only a spherical cytoplasmic body. If the feces be kept for 24 to 48 hours 2 sporoblasts will form within. Within these, in turn, 4 sporozoites will form. These, however, are rarely seen.

*Isoospora hominis* is very rare, producing oocysts 15 by 10 micra.

**Blastocystis Hominis.**—This organism is believed to belong to the yeasts or molds. It is very commonly found in feces. In the unstained preparation it appears as a colorless, round, sometimes oval cell, measuring 5 to 15 micra in diameter. It consists of a central clear body surrounded by a narrow rim of cytoplasm which contains a number of refractile spots or nuclei. These bodies are often mistaken for cysts of protozoa. Their significance is not fully determined. They are not considered pathogenic, and are seldom reported unless they are present in unusually large numbers (Figs. 241 and 251).

## METHODS OF EXAMINATION FOR THE OVA AND LARVAE OF THE HELMINTHES (FLUKES, TAPEWORMS AND ROUNDWORMS)

The laboratory diagnosis of the majority of the helminthes is accomplished by the finding of ova or embryos in the feces. The finding of even one typical ovum is sufficient to establish a diagnosis.

In the routine examination of the feces for ova, the following order should be adhered to:

1. Direct smears of the feces. If negative,
2. Examination by a concentration method. If still negative,
3. Examination by culture (when applicable).

**Collection.**—1. In examinations for the ova and larvae of the helminthes, stools should be collected in clean, dry containers and should not be mixed with urine. Most of their ova are identifiable for several days after passage, and usually a small, representative sample of feces is sufficient. Specimens obtained by enemata will often be positive for ova or segments of tapeworms when ordinary stools are negative. In feces secured by purging, the whole or fragments of adult worms and ova may be readily obtained. If vermifuges have been employed, all the feces for at least 24, and preferably 48, hours should be preserved for examination as a check on treatment. As a rule the feces should be examined for ova and larvae at intervals after treatment and over long periods in strongyloidiasis and clonorchiasis.

2. Enterobiasis or oxyuriasis is best diagnosed by using scrapings of the anal and perianal regions. Although various blunt curettes and anal swabs have been employed, the most efficient is the NIH cellophane swab described by Hall. This consists of a small square of cellophane wrapped about the tip of a glass rod and held in place by a wide rubber band (Fig. 252). The prepared swab is inserted in a perforated stopper and placed in a test tube to prevent loss of material after collection and to protect the handler from accidental infestation. The swab is used with a firm stroking motion, directed outward from the anal opening, parallel to and entering the folds of skin of the entire perianal region. The best time for swabbing is early morning before defecation and bathing. Eggs of helminthes and sometimes the entire worm are picked up by the swab. At least 7 swabs, procured on different days, should be examined before a negative diagnosis is warranted, although most cases will be detected by the first examination. This method is stated to be far more effective than examinations of the feces.

3. At times the duodenal contents will reveal parasites when ova or larvae have not been detected in the feces. It is possible that duodenal or intestinal lavage (deRivas) with warm magnesium sulfate solution will dislodge helminths or their ova, which may subsequently be found in the feces.

4. In the *shipment of feces* (unfixed specimens), the following precautions should be taken: (a) The size of the specimen should not be more than one-tenth of the capacity of the containing bottle, so as to allow for the formation and expansion of



FIG. 252.—THE NATIONAL INSTITUTE OF HEALTH CELLOPHANE SWAB FOR COLLECTING *Enterobius* OVA

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)



gases; (b) the cork should be firmly tied in the neck of the bottle; (c) the label should have the name of the patient, the date passed, and how the specimen was obtained (with or without purge). Where quantitative examination should be made, the size of



FIG. 253.—TAKING A SAMPLE OF FECES (BENBROOK)

the specimen in grams should be given. This may easily be obtained by weighing the specimen before and after it is placed within the receptacle.

**Thin Smear Method for Ova.**—1. Thoroughly emulsify a representative bit of feces or mucus in a few drops of water or saline solution on a clean slide. Usually the surface of the feces will yield the most ova.

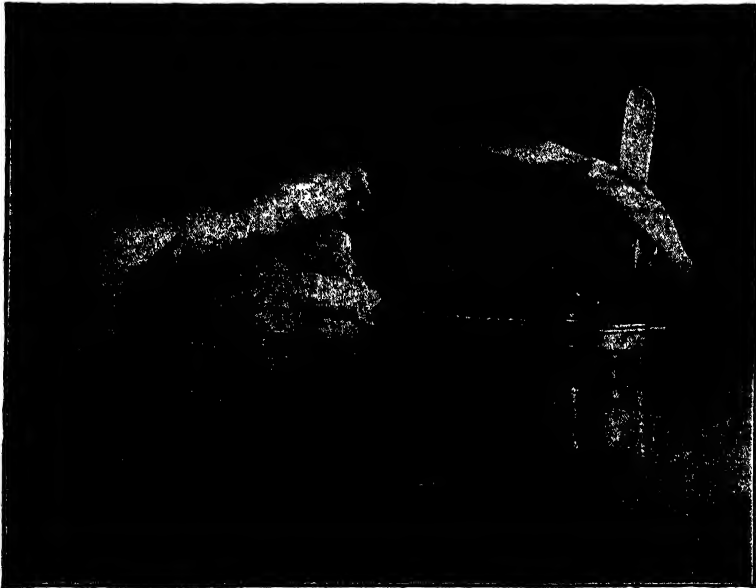


FIG. 254.—STRAINING FECES (BENBROOK)

2. Spread thinly and evenly and cover with a coverglass. A satisfactory film is slightly opaque and allows newsprint to be read through it.

3. Examine the film systematically in meander lines. At least 3 films should be examined before a negative report is made.

4. Iodine-stained preparations are advisable to avoid missing the cysts of intestinal protozoa, such as amebae, in routine examinations.

**Thick Smear Method for Ova.**—Thick, dry fecal smears are prepared about ten times as concentrated as thin smears and cleared with cedar, wintergreen or paraffin oil. The method is stated by Faust to be excellent for the ova of *Ascaris*, *Taenia*, *Trichuris* and *Hymenolepsis*. Other ova may not be clearly visible while others are unidentifiable because of shrinkage.

**Concentration Methods for Ova.**—*Shearer's Method (Benbrook Modification)*: —1. Pick up at least 1 gram of feces using a wooden tongue depressor, and place in sufficient water to liquefy it. Do not use too much water.

2. Thoroughly mix the feces with the water (Fig. 253).

3. Coarse particles may be removed if necessary by straining (Fig. 254).

4. Fill a test tube or centrifuge tube nearly half full of the fecal mixture (Fig. 255).

5. Add to the above an equal quantity of sugar solution prepared as follows.

Granulated sugar ..... 1 lb.

Water ..... 12 oz.

Dissolve the sugar in the water, by immersing the bottle in hot water. Add 1 per cent phenol as a preservative.

6. Mix by slowly inverting the tube several times.

7. Centrifuge the tube containing the mixture for about 3 minutes at moderate speed, 1500 to 2000 revolutions per minute. Centrifuging may be omitted if the tube is allowed to stand 12 to 24 hours.

8. Remove the tube from the centrifuge to a test tube holder without shaking.

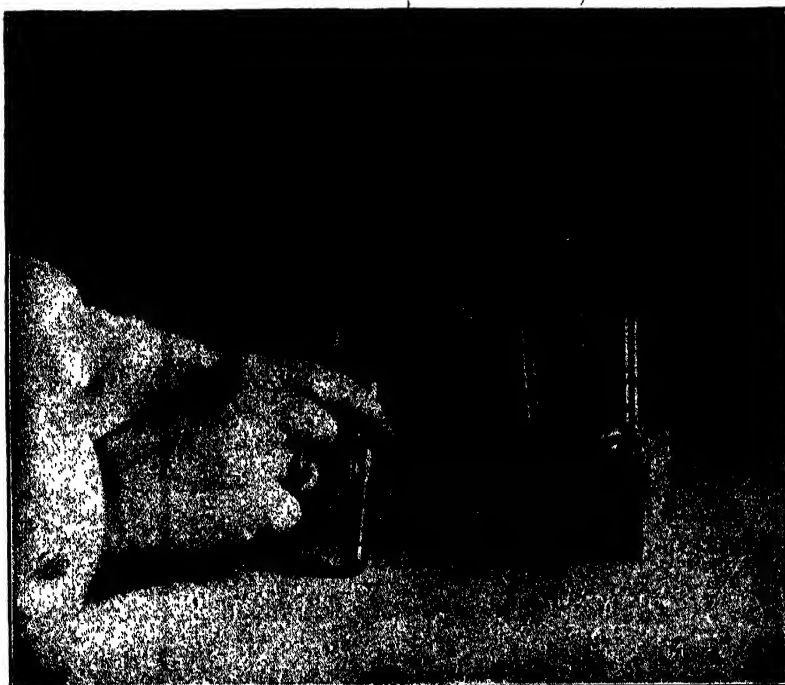
9. Lift off the surface layer of fluid (which now contains the eggs) from the tube by means of a headed glass rod prepared as follows:

Heat one end of a 6 inch length of 5 millimeter glass rod until it is soft enough to be "headed" against a cold metal object. The head portion should be just slightly less in diameter than the inside of the tubes used (see Fig. 256). A heavy glass rod, slightly smaller than the inside of the tube, may be used in place of the headed rod. The rod should be slowly lowered into the tube and the instant full contact is made with the liquid withdraw the rod quickly, bringing with it a large drop usually carrying a number of ova.

10. Transfer the drop from the rod to a microscope slide by gently rotating the rod in the center of the slide. A second or third drop may be added to the first to obtain sufficient material to fill in under a micro cover slide.

11. Carefully lower a micro cover slide on the drop without pressure.

12. Examine the slide under the low power of the microscope. For best results, bright illumination should be obtained by adjusting the mirror and condenser and then modified by closing the diaphragm opening. The microscope should be vertical, not inclined, and the cover-slide area should be searched in a systematic manner. A mechanical stage is recommended.



**FIG. 255.—FILLING CENTRIFUGE TUBE WITH FECAL SUSPENSION (BENBROOK)**



**FIG. 256.—LIFTING SURFACE WITH A BEADED GLASS ROD (BENBROOK)**

13. When parasite eggs are seen, the high dry power should be used for identification.

**Brine-Flotation Method of Kojoid and Barber.**—This method is especially useful when a large number of examinations must be made, particularly for the ova of nematodes and cysts of amebae. According to McDonald, it is unsatisfactory for trematode eggs, which fail to rise to the surface.

1. A large fecal sample is thoroughly mixed with about twice its volume of saturated solution of table salt in a paraffined paste-board cup or a small beaker.

2. A lightly compressed circular disk of No. 1 or No. 0 steel wool about  $\frac{1}{8}$  to  $\frac{1}{4}$  inch thick is then placed in the cup and pushed to the bottom. This carries down all coarse particles.

3. The fluid is allowed to stand for 1 hour, during which time the ova rise to the surface.

4. Finally, the surface film is looped off with a wire loop about  $\frac{1}{2}$  inch in diameter, placed on a slide, and examined without a coverglass. The objective should be focused on the surface of the fluid.

**Willis-Malloy Method.**—1. Dilute and thoroughly emulsify about 1 gm. of feces with 10 to 20 volumes of a saturated solution of sodium chloride in water.

2. Transfer to a glass cylinder about 2.5 cm. in diameter. Use enough brine to form a meniscus.

3. Carefully place a clean fecal slide (75 x 37 mm.) on the meniscus and allow it to remain for 10 to 30 minutes (no longer, in order to avoid disintegration of ova).

4. Remove slide, invert and examine.

**Faust's Zinc Sulfate Centrifugal Flotation Method.**—1. Make a suspension by using 1 part of feces (about size of a pecan) with about 10 parts of lukewarm tap water.

2. Strain about 10 cc. through one layer of *wet* cheesecloth into a test tube.

3. Centrifuge 45 to 60 seconds at top speed. Pour off supernatant fluid, add 2 or 3 cc. of water, break up sediment by shaking or tapping, and add more water to fill tube. Centrifuge in same manner.

4. Repeat (usually 3 or 4 times) until supernatant fluid is clear.

5. Pour off last supernatant fluid, add 3 or 4 cc. of 33 per cent zinc sulfate solution (specific gravity 1.180), break up packed sediment and add more zinc sulfate solution to within about  $\frac{1}{2}$  inch of the rim.

6. Centrifuge for 45 to 60 seconds at top speed.

7. With a bacteriological loop remove several loopfuls of material floating in the surface film to a clean slide, add 1 drop of D'Antoni's iodine stain, and agitate the preparation manually to insure uniform staining. Mount with coverglass and examine. This method detects light infestments of both ova and larvae and also protozoan cysts.

**Caldwell and Caldwell's Ova Counting Method.**—1. In a test tube or flask calibrated at 40 cc., place 4 gm. (approximately 4 cc.) of the stirred fecal specimen.

2. Add 4 cc. of antiformin and thoroughly mix with a glass rod.

3. Leaving the rod in the tube or flask, allow the suspension to stand at room temperature or in incubator for 1 hour or more.

4. Add 32 cc. of sugar solution (750 gm. of sugar in 1000 cc. tap water) and stir thoroughly.

5. Insert a pipet to the bottom and bubble air through the suspension for mixing purposes.

6. Withdraw 0.1 cc. of suspension from the middle portion immediately after bubbling.

7. Place the sample on a slide and spread with a pointed instrument into a small, flat, square or rectangular film. No coverglass is needed.

8. Count ova and multiply by 100 to obtain ova per gram of feces.

9. The use of a 0.2 cc. specimen will register the presence of 50 ova per gram and sometimes fewer than that number. In this case the factor is 50.

**Cultural Methods.**—*For Schistosoma (after Simmons).*—1 Dilute a portion of specimen with water in large-mouth sterile test tube or flask.

2. Allow to stand at room temperature overnight.

3. Examine a drop of fluid taken from directly beneath the pellicle on the water with pipet having a large aperture. The miracidia will escape from the egg and may be found immediately below the pellicle on the water.

*For Hook-worm (Modified from Stitt).*—1. Smear a generous portion of stool suspected of containing the hook-worm ova upon a filter paper small enough to be contained within a Petri dish.

2. Place 2 microscopic slides on the bottom of the sterile Petri dish, and lay the filter paper containing the feces on top with the buttered surface up. Sufficient tap water is added to just cover the filter paper.

3. Cover and allow to stand at room temperature for 5 or 6 days.

4. Examine fluid for the hook-worm embryos.

### AIDS TO THE IDENTIFICATION OF THE MORE COMMON OVA ENCOUNTERED IN THE FECES OF MAN

**Operculum.**—*Large*, over 50 micra, ova do not contain embryo in feces

Ellipsoidal, rounded at both poles, large size:

135 x 80 micra ..... *Fasciolopsis buskii*

140 x 80 micra ..... *Fasciola hepatica*

Abundant in sputum, less frequent in feces, "

medium size:

95 x 55 micra ..... *Paragonimus westermanii*

Oval, small size:

70 x 45 micra ..... *Diphyllobothrium latum*

*Small*, under 50 micra:

Ovum widened at nonoperculated pole, giving shape  
of an electric light bulb

29 x 16 micra ..... *Clonorchis sinensis*

No widening at nonoperculated pole,

30 x 11 micra ..... *Opisthorchis felinus*

Ovoid, with slight thickening at opercular rim,

29 x 16 micra ..... *Heterophyes heterophyes*

**No Operculum.—Wall Opaque.**

Wall thick and smooth

Ovum elongated

Terminal spine 140 x 50 micra. Present in urine,

rare in feces ..... *Schistosoma hematobium*Lateral spine, 150 x 50 micra..... *Schistosoma mansoni*Minute lateral knob, 85 x 60 micra... .. *Schistosoma japonicum*Ovum rounded, with radical striae, hexacanth  
embryo

Ovum spherical

35 x 35 micra ..... *Taenia solium*33 x 33 micra ..... *Echinococcus granulosus*Ovum ovoid, 35 x 25 micra..... *Taenia saginata***Wall Transparent**

Wall thick

Barrel-shaped, clear plugs at poles, 52 x 23 micra.. *Trichocephalus trichiura*  
(*Trichuris trichiura*)Globular with hexacanth larva, 40 x 36 micra.... *Dipylidium caninum*

Wall thin.

Two to four blastomeres

70 x 38 micra ..... *Necator americanus*60 x 40 micra ..... *Ancylostoma duodenale*Bulge on one side, flat on other, 55 x 25 micra.... *Enterobius vermicularis*Three membranes, globular, small hexacanth em-  
bryo, 40 x 35 micra..... *Hymenolepis nana***Wall ornamented**

Mammillated, brown,

60 x 45 micra ..... *Ascaris lumbricoides*

Ellipsoidal, three embryonic envelopes, shallow loz-

enge-shaped depression, 90 x 50 micra..... *Macrocanthorhynchus*  
*hirudinaceus***METHODS FOR THE IDENTIFICATION OF THE FLUKES  
(TREMATODA)**

The flukes or trematoda are flat, leaf-like worms, nonsegmented, attaching themselves to the mucous membranes by suckers. The oral sucker, located at the anterior end, leads into an esophagus which soon divides to form two caeca. The ventral sucker is found anterior to the oral sucker, its position varying with the species. Ovaries and testes are present. The vitellaria are usually prominent lateral structures.

**Fasciolopsis Buskii.**—This is a large fluke infesting the small intestine and producing fasciolopsiasis. Infestation is common in the Far East, particularly China. The fluke is a large oval-shaped organism, 20 to 75 mm. in length, 8 to 20 mm. in width and 2 to 3 mm. thick, opaque flesh color. The oral sucker is small. The acetabulum is large and located just posterior to the oral sucker. The testes are branched and posterior. The vitelline glands are found along the sides of the body.

The *ova* are large, 135 x 80 micra, brown in color, oval in shape, with a delicate operculum at one end. The contents are granular without embryo (Fig. 257).

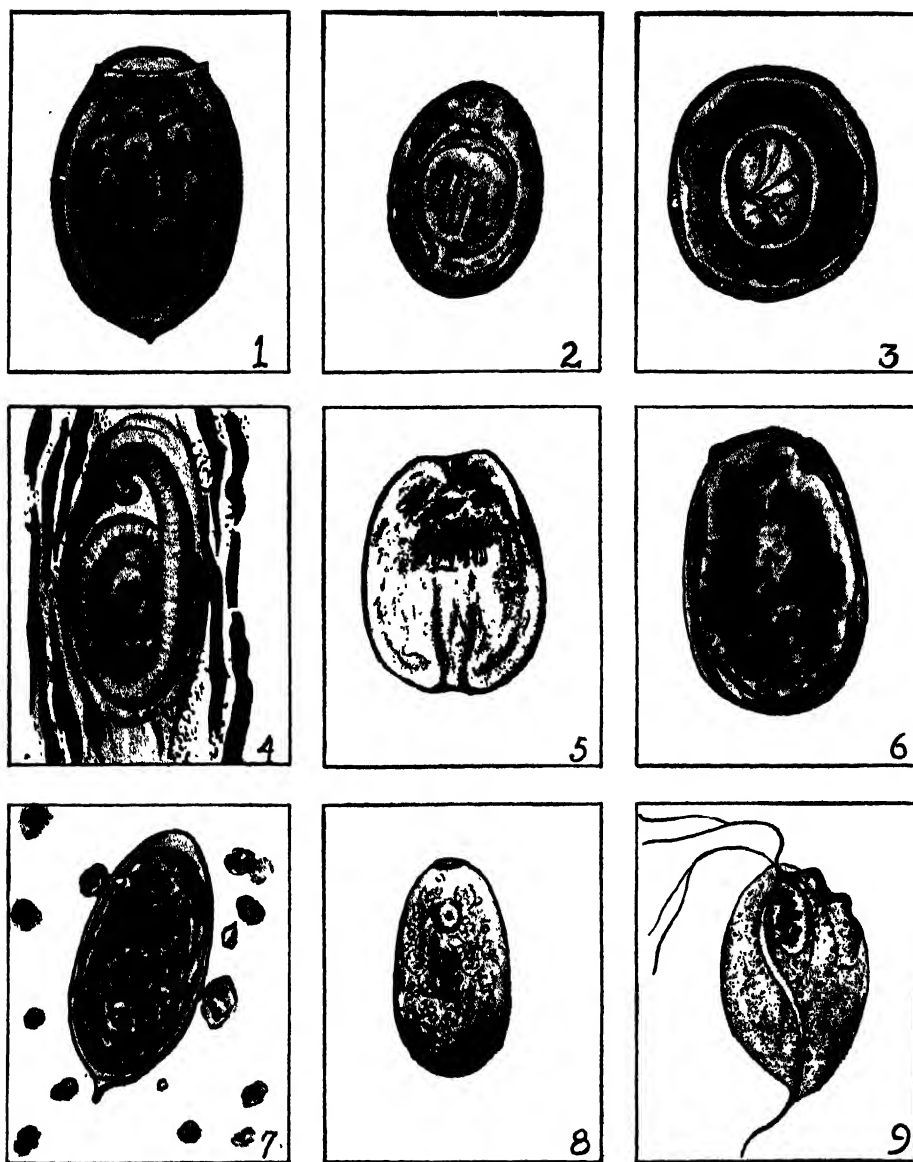


FIG. 257.—OVA OF ANIMAL PARASITES

1, Ovum of *Diphyllbothricum latum*; 2, ovum of *Hymenolepis nana*; 3, ovum of *Hymenolepis diminuta*; 4, encysted larvum of *Trichnella spiralis* in muscle; 5, a scolex of *Echinococcus granulosus* from a liver abscess; 6, ovum of *Paragonimus westermanii* in sputum; 7, ovum of *Schistosoma haematobium* in urine; 8, ovum of *Fasciolopsis buski*; 9, *Trichomonas vaginalis*. (From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

**Fasciola Hepatica.**—This fluke infests the biliary tract, producing fascioliasis. About one-fourth of cases of the disease have been reported from Cuba. It commonly infests sheep and other herbivorous animals. It is about 30 mm. in length and of an opaque flesh color (Fig. 258).

The fluke is leaf-like in shape, anteriorly terminating in a projecting cone which ends in the oral sucker. The acetabulum is located at the base of the anterior cone-like projection. Two-branched intestinal caeca are present. The testes and ovary are finely branched.

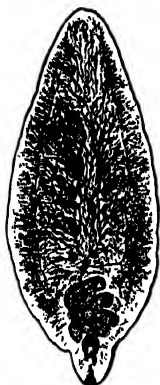


FIG. 258.—*Fasciola Hepatica*  $\times 5$ .

(From Wood, *Chemical and Microscopical Diagnosis*, D. Appleton and Co., New York.)

The ova are  $140 \times 80$  micra, operculated and similar to those of *F. buskii*.

**Clonorchis Sinensis** (*C. endemici*.)—This fluke invades the ducts of the liver of man. The infestation is of importance in the Far East, particularly North China. The fluke measures 10 to 20 mm. in length, 3 to 5 mm. in width. It tapers at the anterior end. Its cuticle is smooth and the fluke is almost transparent. The acetabulum is located one-fourth of the length of the body from the anterior end. Two dilated caeca are found. The branched testes are located in the posterior third of the body. Lobed ovaries in the mid-portion (Fig. 259).

The ova measure 29 by 16 micra, light brownish color. The shape is said to resemble an electric light bulb with a flattened operculum at the smaller end.

**Opisthorchis Felineus.**—A liver fluke of dogs and cats, occasionally infesting man. A few cases are found throughout Europe and Asia. The fluke is elongated and resembles *C. sinensis*. It is 7 to 12 mm. in length. It is differentiated from *C. sinensis* by the posterior location of the lobed testes, one on either side of an excretory duct.

The ova measure 30 by 12 micra, and are similar to those of *C. sinensis*.

**Paragonimus Westermanii.**—This infestation is of importance in the Far East and in Central and South America. The organism is flat, oval shaped, reddish brown in color, 12 by 6 mm. and 5 mm. in thickness. The ventral sucker is almost centrally located. The lobed testes are located in the posterior portion on either side of the excretory duct. The vitellaria extend laterally along the length of the body.

The ova are operculated,  $95 \times 55$  micra in size. They are most frequently found in the sputum, but may be swallowed and appear in the feces (Fig. 257). About two-fifths of patients show ova in the feces.

**Schistosoma Hematobium.**—This infestation is principally found in parts of Africa and the Mediterranean basin. This fluke has sex differentiation, and is long

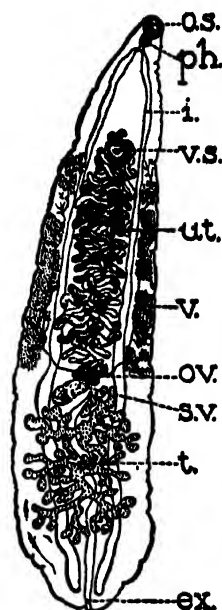


FIG. 259.—*Clonorchis sinensis*, SHOWING INTERNAL STRUCTURE

o.s., oral sucker; ph., pharynx; i., intestine; v.s., ventral sucker; ut., uterus; v., vitellaria; ov., ovary; s.v., vesicula seminalis; t., testis; ex., excretory duct. *Bythinia striatula*, var. *japonica* is first intermediate host. ( $\times 6$ ). (From Ward, *Abt's Pediatrics*, W. B. Saunders Co., Philadelphia.)



and round in shape. The male measures 10 by 15 mm. and has a ventral groove into which the slender female, 20 mm. in length lies. Both the male and female fluke are equipped with ventral and oral suckers. The male is considerably larger in diameter than the female.

The ova measure 140 by 55 micra in diameter, are oval and contain a fairly well-developed miracidium. The ova are deposited in the venous plexes about the urinary

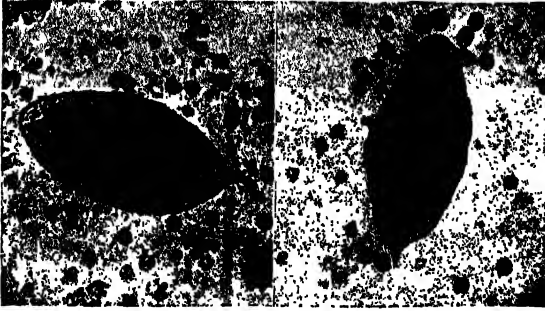


FIG. 260.—OVA OF *SCHISTOSOMA HAEMATOBIMUM* WITH PUS CORPUSCLES IN URINE (PHOTOGRAPH,  $\times 250$ )

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

bladder. The ova work their way through the tissues, appearing in the urine, and in smaller numbers, in the feces. The ova are equipped with terminal spines (Fig. 260).



FIG. 262.—OVUM OF *SCHISTOSOMA JAPONICUM*.  $\times 460$  (MORRIS)

(From Morris, *Clinical Laboratory Methods*, D. Appleton and Co., New York.)

contained within. A tiny easily-overlooked spine is present (Fig. 262).

#### *Schistosoma Mansoni*.—

The infestation is chiefly confined to parts of Africa and Northern South America. The male fluke measures 10 to 12 mm. in length, the female 12 to 16 mm. in length, and considerably less in diameter than the male.

The eggs are laid in the small veins of the intestine, reaching the feces by penetrating the wall. The ova measure 150 by 65 micra, and contain well-developed miracidia. They are identified by the large lateral spine (Fig. 261).

*Schistosoma Japonicum*.—This fluke is chiefly found in the Far East. The male measures 12 to 20 mm. in length, the female 15 to 26 mm. in length. The female is smaller in diameter than the male.

The flukes deposit the eggs in the small veins of the intestine and reach the feces by penetrating the wall. They are 85 by 60 micra in size. A well-developed miracidium is con-



FIG. 261.—OVUM OF *SCHISTOSOMA MANSONI*  $\times 460$  (MORRIS)

(From Morris, *Clinical Laboratory Methods*, D. Appleton and Co., New York.)

## METHODS FOR THE IDENTIFICATION OF THE TAPEWORMS (CESTODA)

Tapeworms are flat, long, ribbon-like worms made up of a number of segments. The head or scolex is extremely small, about 1 mm. in diameter, and is provided with sucking disks, and in some species hooklets for attachment. The segments or proglottids develop from the head end and become progressively larger as they mature. The size of the worm varies according to the species.

The *proglottids* are segments which are sexually mature. A tapeworm might be considered as a colony of flukes attached end to end, each segment representing a complete sexual unit, and containing both testes and ovaries. Nutrition is absorbed directly through the cuticle. The size of the worms vary from less than an inch to over 20 feet.

Man is the ultimate or definitive host for most tapeworms, the eggs being passed in the feces. These are ingested by animals or fish who develop the encysted larvae in their muscles. Man acquires the disease by eating meat or fish containing the encysted embryos. In rare instances he may act as the intermediate host, by ingesting the ova (somatic taeniasis).

**Taenia saginata (Beef Tapeworm).**—*T. saginata* is a very common infestation of man, and is acquired by eating beef containing the encysted embryos (*Cysticercus bovis*).

The adult worm is from 10 to 25 feet in length, and contains from 1000 to 2000 segments. The scolex or head measures from 1 to 2 mm. in diameter. It is pear-shaped, and has 4 sucking disks arranged around the head laterally. No rostellum or hooklets are present.

Mature proglottids are longer than wide, about  $\frac{1}{2}$  inch wide, and contain a uterus with 18 to 30 lateral branches. The genital pore is lateral and irregularly alternate (Fig. 263). These can easily be seen by placing the segment between 2 slides and examining by transmitted light, with the naked eye or a hand lens.

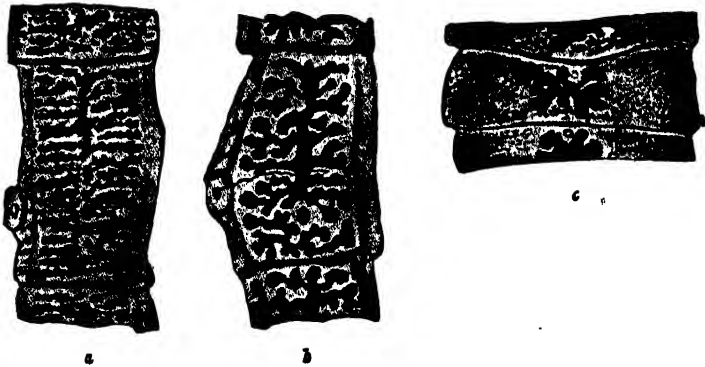


FIG. 263.—SEGMENTS OF THE THREE LARGE TAPEWORMS OF MAN, SHOWING ARRANGEMENT OF UTERUS

a. *Taenia saginata*; b. *Taenia solium*; c. *Diphyllobothrium latum* (×5). (From Cambridge, *The Feces of Children and Adults*, William Wood & Co., New York.)

The *ova* are about 35 micra in diameter, round or oval in shape. The outer covering (embryophore) is radially striated. Within is a hexacanth embryo, the hooklets of which may be recognized as 6 tiny paired lines (Figs. 264 and 265).

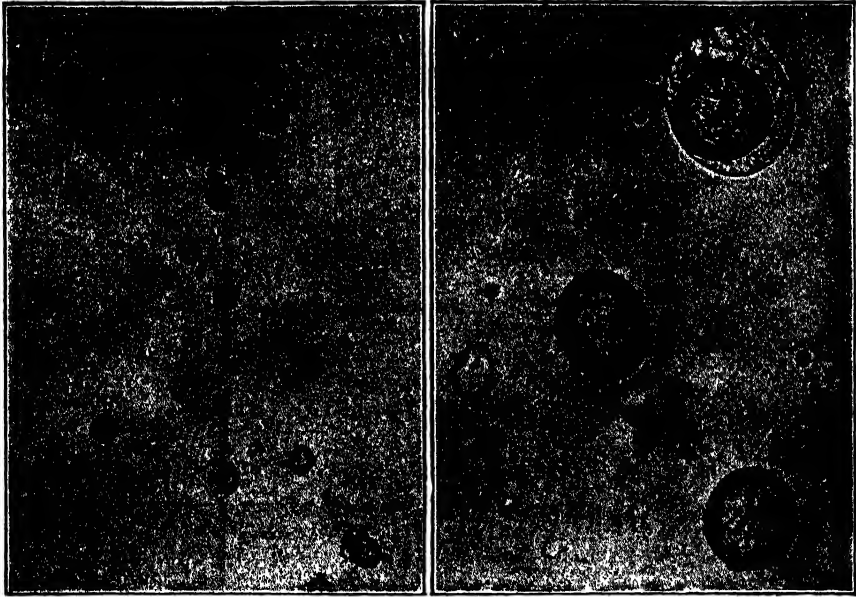


FIG. 264.—OVA OF *TAENIA SAGINATA* (BENBROOK)  $\times 100$  and  $\times 400$

The laboratory diagnosis is made by the finding of the ova or segments in the feces.

**Taenia Solium (Pork Tapeworm).**—*T. solium* is an important tapeworm of man, but less important in this country than *T. saginata*. Man may suffer from both the infestation of the adult form, or, rarely, the larval stage.

The adult worm is 7 to 15 feet in length, and contains from 800 to 1000 segments. These segments are longer than they are wide. The scolex or head is globular, and about 1 mm. in diameter, and is provided with 4 sucking disks laterally, and a rostellum, around which are arranged 28 hooklets. The mature *proglottids* contain a uterus with 8 or 9 lateral branches. The genital pore is lateral and irregularly alternate (see Fig. 263).

The *ova* are practically identical with *T. saginata*. They are about 35 micra in diameter, and brownish in color. The outer covering is radially striated. Within, is a small hexacanth embryo (oncosphere) with 3 pairs of hooklets.

Diagnosis is made by finding of the ova or segments in the feces. When man is the seat of *somatic taeniasis* (the embryo stage) the larvae, the cysticerci cellulosae, may be found encysted in the muscles and other organs of the body. The encysted larvae measure 5 to 10 mm. in diameter, and their laboratory diagnosis is accomplished by examining excised bits of muscle pressed between 2 pieces of glass, as in examination for *Trichinella spiralis*.

**Hymenolepis Nana (Dwarf Tapeworm).**—This tapeworm is a very common infestation, particularly of children.

The adult worm is small, measuring 1 to 2 inches in length, and is made up of about

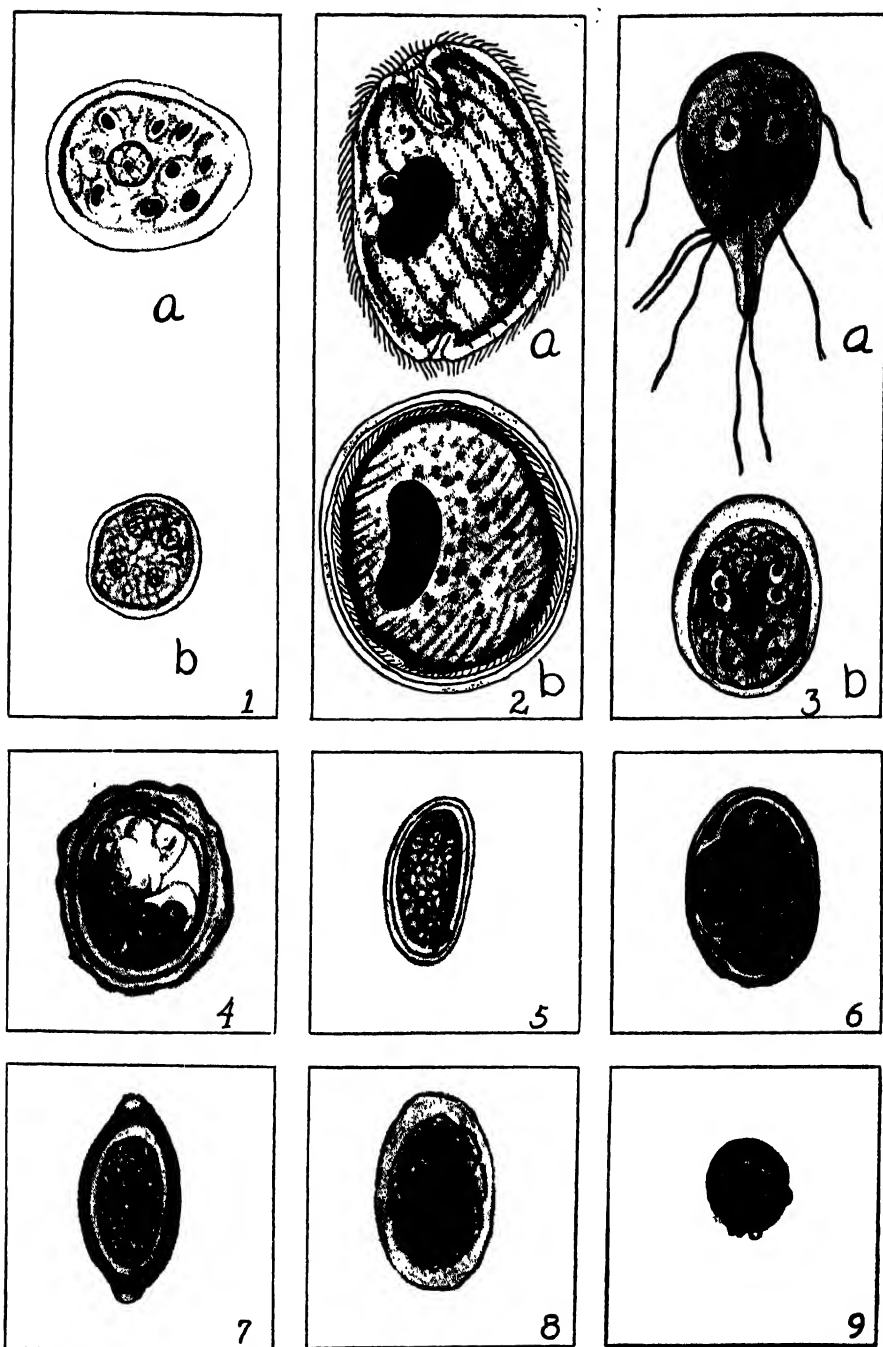


FIG. 265.—TROPHOZOITES, CYSTS AND OVA OF ANIMAL PARASITES

1, Trophozoite (a) and cyst (b) *E. histolytica*; 2, trophozoite (a) and cyst (b) of *Balantidium coli*; 3, trophozoite (a) and cyst (b) of *Giardia lamblia*; 4, ovum of *Ascaris lumbricoides*; 5, ovum of *Oxyuria vermicularis* (*Enterobius vermicularis*); 6, ovum of *Necator americanus*; 7, ovum of *Trichuris trichiura* (*Trichocephalus trichiuris*); 8, ovum of *Strongyloides stercoralis*; 9, ovum of *Taenia saginata*. (From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

200 segments. The scolex is very tiny, about  $\frac{1}{3}$  of a mm. in diameter, has 4 sucking disks laterally placed, and has a rostellum anteriorly. Around it a row of from 24 to 30 hooklets are arranged.

The mature *proglottids* are less than a mm. in width. They are identified by their small size, 3 testes to each segment, and an irregular sac-like uterus which contains many ova.

The ova are rounded or globular in shape, about 40 micra in diameter and quite transparent. *The light must be considerably reduced in order to reveal this ovum under the microscope.* A hexacanth embryo is centrally located with 3 surrounding membranes. The space between the outer (vitelline membrane) and the middle (embryophore) is filled with a semi-solid material in which waxy, refractile filaments extend from each pole in a most characteristic manner (Fig. 257).

The diagnosis is made by finding the ova in the feces. The segments may be accidentally picked up in the microscopic examination, but are too small to be detected in the gross examination of the feces.

**Diphyllobothrium Latum (Fish Tapeworm).**—This tapeworm is of importance in certain parts of the world where fish is eaten, insufficiently cooked or raw.

The adult worm measures from 3 to 10 meters, 10 to 34 feet in length, and has approximately 4000 segments. The scolex is almond-shaped, about 2 to 3 mm. in length, and has 2 lateral sucking grooves. There is no rostellum and no hooklets. The mature segments may be identified by the rosette-shaped centrally placed uteri (see Fig. 263). The segments are wider than they are long. The genital pore is in the middle of the segment.



FIG. 266.—OVA OF *DIBOTHRIOCEPHALUS LATUS*

(From Wood, *Chemical and Microscopical Diagnosis*, D. Appleton and Co., New York.)

The ova are 70 by 45 micra. They are oval in shape, somewhat brownish in color, and have a small operculum. There is no embryo within (Figs. 257 and 266).

Laboratory diagnosis is established by finding the ova or segments in the feces.

**Taenia Echinococcus (Echinococcus Granulosus), Dog Tapeworm.**—This infestation in man depends upon the close association between man and dogs in cattle and sheep raising countries. When man is infested he becomes the intermediate host for the parasite, the adult sexual phases taking place in the dog.

The adult worm as found in the dog is small, measuring 3 to 5 mm. in length, and consists of head and three segments. The scolex has four suck-

ing disks, a rostellum and 38 hooklets.

The ova are spherical, about 33 micra in diameter, and are similar in appearance to those of *Taenia solium* and *Taenia saginata*. When the eggs are ingested by man the embryos produce echinococcic cysts in various organs, particularly the liver.

The cyst is formed from the body of the embryo parasite. It enlarges, and by invaginations of the germinal layer, produces daughter cysts within. These develop secondary and tertiary invaginations, finally forming scolices or heads attached to the

wall by a pedicle. The scolices have four sucking disks and a number of hooklets (Fig. 257). Thousands are formed in each cyst.

Diagnosis cannot be made from the feces. Diagnostic puncture of the cyst is attended with great danger of spreading the infection and of shock resulting from the leaking out of cyst fluid. If such puncture be made, however, the diagnosis is accomplished by finding the scolices, or in old cysts where the scolices have disintegrated, the finding of tiny chitinous hooklets.

Other diagnostic laboratory methods are the complement fixation, precipitin, and skin tests.

**Dipylidium Caninum.**—This tapeworm is commonly found in dogs and cats. It occasionally infests man.

The adult worm is from 4 to 20 inches in length, and is made up of about 20 segments. The scolex is about  $\frac{1}{3}$  mm. in diameter, has 4 sucking disks, and a retractile rostellum projecting from the anterior end with 3 or 5 rows of spines or hooklets. The mature segments are elliptical in shape, and have a genital pore on each side.

The *ova* are 40 micra in diameter, slightly oval in shape, and resemble the *ova* of *Hymenolepis nana*, except that the hexacanth embryo is much larger, and the surrounding membranes are thus closer together.

## METHODS FOR THE IDENTIFICATION OF THE ROUNDWORMS (NEMATODA)

The sexes are separate in all the nematodes. They constitute a group of important infestations of man in the United States and elsewhere.

**Ascaris Lumbricoides.**—The adult male is about 23 cm. in length and 0.3 cm. in diameter, the female 33 cm. in length and 0.5 cm. in diameter. They somewhat resemble the earthworm, although not segmented. They are reddish or yellowish in color when freshly passed.

The *ova* are easily recognized, measuring 60 by 45 micra, and are covered by a coarsely mammillated albuminous covering. Beneath this is a clear transparent shell enclosing an unsegmented protoplasm (Figs. 265 and 267).

The laboratory diagnosis is made by finding the *ova* or parasites in the feces.

**Enterobius Vermicularis (Oxyuris Vermicularis), the Pinworm or Seat-Worm.**—The worms are very small, the female measuring from 9 to 12 mm. and the male 3 to 5 mm. When the eggs are ingested, the embryos are developed in the duodenum, the gravid females descending into the rectum and depositing their eggs in the perianal region during sleep of the individual.

When examined from the feces the females have a long straight pointed tail. The males have a ventral curve, the tail showing a spiculum near the end. Both have a prominent bulbous esophagus. In the female the anus opens at the junction of the middle and posterior third of the body. The vulva opens at the junction of the anterior and middle third. In the male the anus opens just posterior to the spine in the tail. When the worm is mature the whole body appears filled with *ova*.

The *ova* are colorless, oval with one side flattened, measuring 50 micra long by 25 micra wide. Within the shell, when deposited, is a partially developed embryo folded upon itself (Figs. 265 and 268).

Diagnosis is made by examining the scrapings from the perianal region for eggs

and portions of adult worms, or examining the feces following purgation or saline enema. The NIH swab is best for the collection of specimens (see page 571).

**Ancylostoma Duodenale (Old World Hookworm).**—This hookworm is common in southern Europe and Egypt.

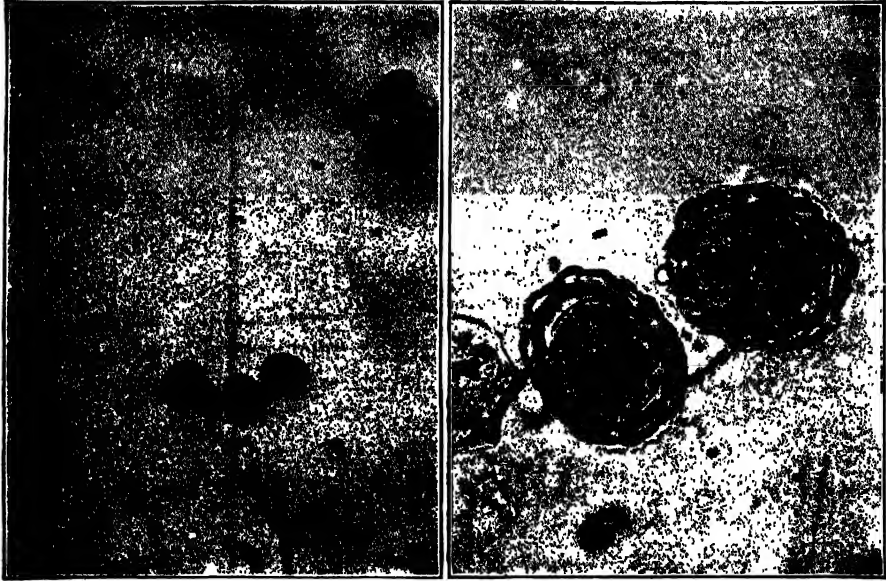


FIG. 267.—OVA OF ASCARIS LUMBRICOIDES (BENBROOK)  $\times 100$  and  $\times 400$

The adult is cylindrical in shape with the head bent dorsally, giving the appearance of a hook; thus the name, uncinaria.

The armature of the buccal cavity consists of 4 claw-like ventral teeth and 2 knob-like dorsal teeth. The female measures 12 to 15 mm. long. The posterior end is bluntly pointed. The vulva is situated at the juncture of the middle and posterior third of the body. The male measures 8 to 10 mm. long, the posterior end expanding into an umbrella-like pouch (copulatory bursa). Within the bursa 2 small fine copulatory spicules can be seen with a low power lens.



FIG. 268.—OVUM OF OXYURIS \* VERMICULARIS  $\times 460$ . (From Wood, *Chemical and Microscopical Diagnosis*, D. Appleton and Co., New York.)

The ova are 60 to 70 micra in length, about 30 micra in width, oval in shape, and colorless. Within a rather wide transparent shell the protoplasm is divided into 2, 4 or 8 cells. *In freshly passed feces it is rare to find more than 4 cells. Embryos are never found in the feces.*

Although the embryos are never found in the freshly passed feces, if the specimen is not examined the embryos may hatch in 24 to 48 hours. The ova likewise may contain more than 4 or 8 cells. Therefore, the importance of immediately examining the fresh feces.

In *Strongyloides stercoralis* infestations the ova are more mature, containing more than 8 or 16 cells, and the embryos are usually passed in the feces.

The rhabditiform larvae measure from 0.25 mm. to 0.5 mm. They are filariform

in shape with a bulbous esophagus. They are differentiated from the rhabditiform larvae of *Strongyloides stercoralis* by the depth of the mouth cavity which is as long as the entire diameter of the larvae measured at the end of the buccal cavity.

The diagnosis is made by finding the ova in the feces by (1) direct smear; (2) concentration methods; or (3) demonstration of the embryos by culturing the feces.

**Necator Americanus (New World Hookworm).**—*Necator Americanus* is common in South and Central Africa, sub-tropical America, southern United States and West Indies. It is similar to *Ancylostoma duodenale* but smaller. The female measures 9 to 11 mm., the male 7 to 9 mm.

The buccal armature consists of 4 central small chitinous plates and 2 well-developed semi-lunar dorsal plates. In the middle of the dorsal wall of the buccal cavity is a prominent conical tooth-like structure.

The vulva is anterior to the middle of the body in the female. The male has a copulatory bursa, somewhat similar to *Ancylostoma duodenale*, except that the dorsal rays are deeply divided, and only 1 spicule is present.

The ova are similar to those of *Ancylostoma duodenale*, but slightly larger, 70 by 40 micra (Figs. 265 and 269).

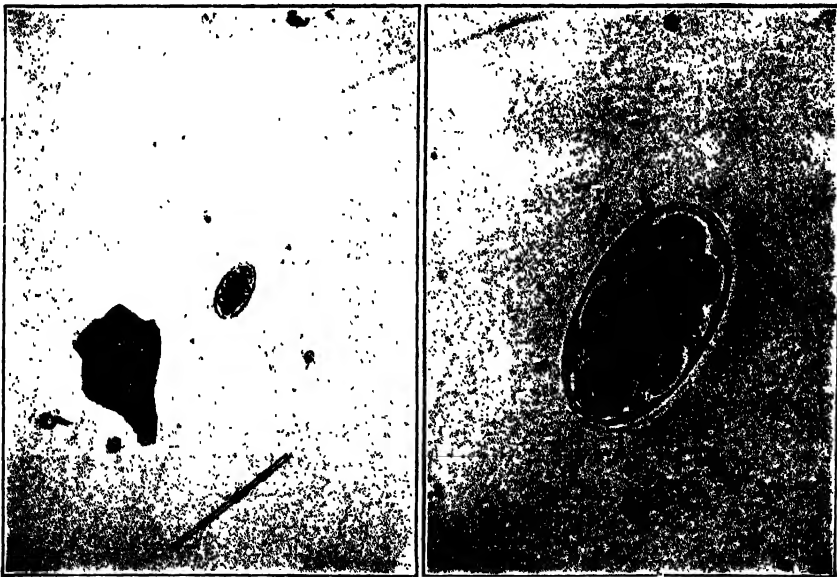


FIG. 269.—OVA OF *NECATOR AMERICANUS* (BENBROOK)  $\times 100$  and  $\times 400$

The rhabditiform larvae are similar to a duodenale.

The diagnosis is the same as for *Ancylostoma duodenale*.

**Strongyloides Stercoralis.**—The geographical distribution of this parasite is much the same as the hookworm. The adult females inhabit the intestine. The females only have been demonstrated.

The ova (Figs. 265 and 270) measure 55 to 32 micra, are oval, thin shelled and transparent, closely resembling the eggs of the hookworm. *They are only present in*



*the feces after purgation, and are usually more mature, showing 8, 16 or 32 cell stages, and are frequently accompanied by embryos.* Hookworm embryos never occur in fresh feces, but may be hatched out in passed feces after 24 to 48 hours.

The eggs hatch in the intestine; the rhabditiform larvae with the eggs pass out in the feces.

The rhabditiform *larvae* measure 250 to 500 micra by 15 to 24 micra. They are differentiated from hookworm larvae by the length of the mouth cavity. The length is one-half the width of the body measured at the posterior end of the cavity. In the hookworm it is much larger, being as long as the entire diameter of the larvae measured at the end of the buccal cavity.

Diagnosis is made by finding the rhabditiform larvae in the fresh feces.

**Trichocephalus Trichiura (Trichuris Trichiura).**—The whipworm is one of the most common intestinal infestations, having a wide geographical distribution. It is called the whipworm because of the appearance of the adult, the thick posterior  $\frac{2}{3}$  resembling a handle, the remaining anterior portion being slender and lash-like. It is chiefly found in the cecum. The male measures from 3 to 4 cm. in length, the female from 3 to 5 cm.

The *ova* measure 52 by 23 micra, and are very characteristic. They are brown in color, and have an inner and outer shell, with a transparent area between, and two knob-like structures at either pole (Figs. 265 and 271).

## METHODS FOR THE LABORATORY DIAGNOSIS OF INTESTINAL MYIASIS

The larvae of several species of diptera (flies) have been reported as found in the feces. This is the result of ingestion of food containing the eggs of flies. Rarely some of the eggs may escape digestion and form larvae which are passed in the feces. Gastro-intestinal symptoms may develop. The infestation is extremely rare. Species of bot flies, *Dermatoibia*, and flesh or meat flies, *Sarcophagga*, have been reported.

The laboratory diagnosis consists of demonstrating the larval forms, the maggots, in the stool.

**Caution.** The diagnosis of intestinal myiasis should never be made unless the examiner is satisfied that there has been no opportunity for flies to lay their eggs or larvae in the specimen between the time of passage and that of examination. The larvae have been known to appear in a remarkably short time.

## MACROSCOPIC AND MICROSCOPIC METHODS FOR ADULT HELMINTHES

1. Place the entire stool specimen in a suitable receptacle, and add a sufficient amount of tap water to make it fluid.

2. Thoroughly mix water with the stool, and pass through a suitable screen (No. 20) to remove fluid. If the fecal matter has been properly broken up, most of it will pass through, leaving the worms or segments on the screen.

3. The material from the screen is now transferred to a clean shallow glass dish or tray (preferably with a black bottom) containing salt solution or tap water. Against this black background the parasite may easily be seen by the use of the

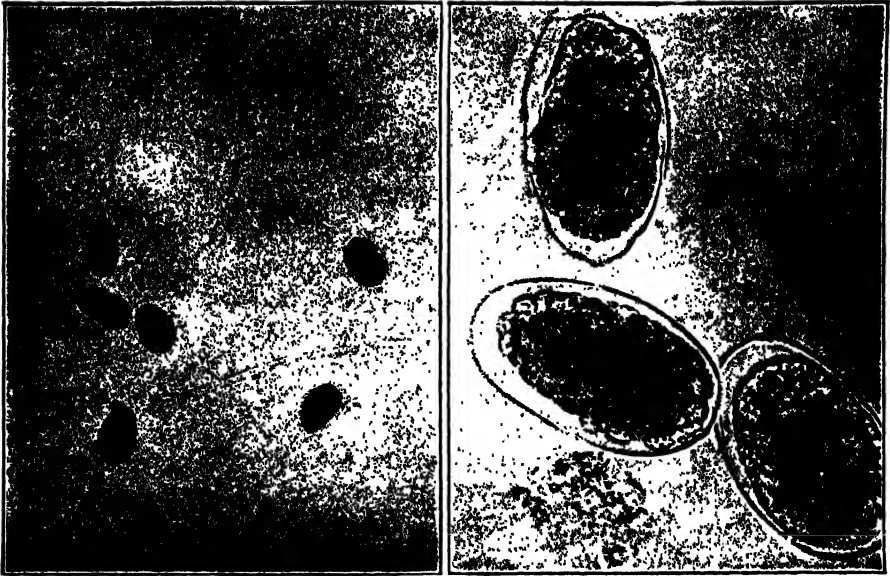


FIG. 270.—OVA OF *STRONGYLOIDES STERCORALIS* (BENBROOK)  $\times 100$  and  $\times 400$

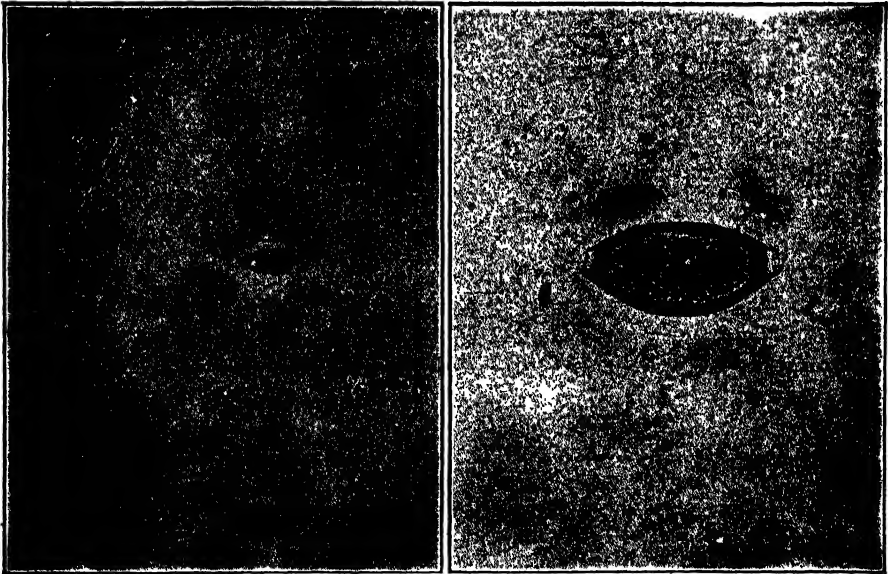


FIG. 271.—OVA OF *TRICHURIS TRICHIURA* (BENBROOK)  $\times 100$  and  $\times 400$

unaided eye. It may be necessary to wash and screen a second time in order to free the parasites from the fecal material.

4. The parasite or segment may now be placed on a microscopic slide, covered with a coverglass, and examined with the naked eye or low power scope.

In examining tapeworm segments and flukes, it is desirable to cover with a second micro slide in place of a coverglass. By pressing the two together the specimen may be flattened, making it less opaque and thus better revealing the anatomical structure within.

Clearing the specimen with carbol-xylol (25 per cent phenol crystals and 75 per cent xylol) will also aid.

5. The specimen is best examined for internal structure by transmitted light. For details of the cuticle, or in the examination for scolices of tapeworms (to show hooklets, suckers, etc.) direct illumination or a combination of the direct illumination and transmitted light will be found advantageous.

6. **In examining tapeworms for the head or scolex**, the following procedure will be found useful:

Arrange a second tray or basin containing salt solution or tap water, adjacent to the one containing the specimen, which has been obtained by washing and screening; see paragraphs 1, 2, 3 and 4 (Fig. 272).

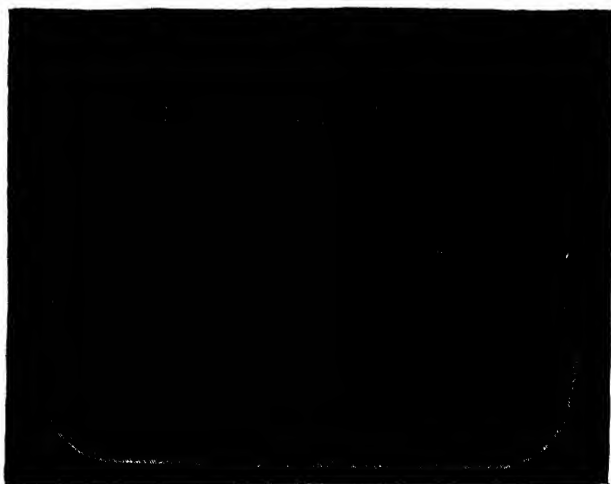


FIG. 272.—APPARATUS FOR THE RECOVERY OF THE HEADS OF TAPEWORMS

(From Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Philadelphia.)

7. With 2 glass rods (about 8 inches long) as fingers feed the tapeworm into the adjacent receptacle, beginning with the larger segments and working toward the smaller.

8. Proceed in this way until the end of the worm or section thereof is reached. Examine with a lens to determine if the head is present. If not, proceed with other segments until all have been removed from the receptacle.

9. Carefully examine any remaining material in the tray for the presence of the head or scolex.

The head is very tiny, about the size of a pin head (1 mm.), the neck and adjacent segments scarcely larger than a heavy thread. They can be easily overlooked if great care is not exercised.

*The finding of the head is of paramount importance to the clinician since the worm will continue to grow as long as the head remains in the intestine.*

## METHODS FOR THE PRESERVATION OF ANIMAL PARASITES AND OVA

**Preservation of Worms.**—*Tapeworms, Cestodes.*—1. Wash the worm free from fecal material and allow to remain an hour or two in fresh clean tap water.

2. After washing, place in 10 times its volume of 3 per cent formaldehyde.

3. After 24 hours the formaldehyde should be changed for permanent storage.

*Flukes, Trematodes.*—1. Wash free from fecal material, and place in 3 per cent formaldehyde.

2. Better results may be obtained by placing the fluke between 2 slides to flatten out, holding the slides together with rubber bands at each end. The slides containing the specimen are then immersed in 3 per cent formaldehyde for 2 hours, after which the slides may be removed and the flattened specimens placed in 3 per cent formaldehyde.

*Roundworms, Nematodes.*—1. Roundworms should be washed free from the feces, and at once placed in salt solution, since tap water will cause them to swell up and burst. They should be killed by placing in hot (70° C.) 3 per cent formaldehyde.

2. After they have been killed, by a few minutes' exposure to the hot formaldehyde, they are placed in 70 per cent alcohol for preservation.

**Preservation of Feces Containing Eggs of Parasites** (after Blacklock and Southwell).—1. Take a small quantity of feces (about 1 cm. in diameter) and mix with sufficient tap water to produce a semi-solid consistency.

2. Add about 200 cc. of 10 per cent formalin (about 90° C.). Mix thoroughly by stirring. Allow to sediment for several hours.

3. Decant overlying, supernatant fluid, and add 200 cc. of 10 per cent formalin to the sediment.

By this method worms, eggs, larvae and intestinal protozoa are fairly well preserved.

# METHODS FOR PARASITOLOGICAL EXAMINATIONS OF THE BLOOD AND TISSUES

## METHODS OF EXAMINATION FOR MALARIA

**Principles.**—1. Malaria is a disease due to the invasion of erythrocytes by protozoa belonging to the genus *Plasmodium*. Four species are of importance: *Plasmodium vivax*; producing tertian malaria, with paroxysms recurring every 48 hours; *Plasmodium malariae*, producing quartan malaria, with paroxysms recurring in 72 hours; *Plasmodium falciparum*, producing "malignant" tertian or estivo-autumnal malaria, with paroxysms recurring irregularly; and *Plasmodium ovale*, a tertian form of the parasite.

2. Final proof of malaria always depends upon finding the plasmodia or their pigments in the blood. In their absence the correctness of the diagnosis is open to question.

3. The parasites are most numerous in the blood 6 to 8 hours after a paroxysm, but they may be found at other times and examinations should be made without any special reference to the occurrence of paroxysms.

4. Blood examinations are also of great value as criteria of cure; all gametocytes should be removed by treatment.

5. The life cycle of the parasites consists of a sexual phase, taking place in the body of a mosquito (anopheles), and an asexual phase within the erythrocytes of man.

6. *Laboratory diagnosis* consists of the examination of (a) fresh wet films of blood; (b) thin stained films of blood; (c) thick stained films of blood; (d) stained films of blood after concentration; (e) stained smears of material secured by splenic puncture in chronic malaria and (f) cultures of the blood (usually omitted in routine examinations).

**Fresh Wet Film Method.**—1. Use perfectly clean and grease-free coverglasses and slides.

2. Puncture a finger or the lobe of an ear and touch a small drop of blood with a coverglass making sure that it does not touch the skin.

3. Carefully lower the inverted coverglass on the center of a slide. After the blood has spread the center of the film should be so thin that it is almost colorless.

4. Examine first with the high dry objectives and, if necessary, with the oil-immersion objective.

5. If it is desired to observe the film over a period of several days, seal the edges of the coverglass with vaselin to prevent evaporation.

**Thin Stained Film Method.**—1. Smears are prepared with coverglasses or slides in the same manner as for differential leukocyte counts, but the films must be so thin that the erythrocytes lie flat and are well separated.

2. Fix and stain with Wright's or Giemsa's stain in the same manner as staining for differential leukocyte counts.

3. Dry and examine with oil-immersion objective.

4. The smears must be well stained for satisfactory results. Unless the nuclei of leukocytes are well stained and have the proper reddish-purple tint due to proper staining of the chromatin, the chromatin of the plasmodia will not be properly stained. Good and poor areas may occur on the same slide.

5. Malaria plasmodia are in the erythrocytes, and no object should be considered as a probable plasmodium unless it is so situated.

6. With Wright's or Giemsa's stain the chromatin of the parasite will take on a ruby red color, the protoplasm of the organism a sky-blue (pale blue), the pigment a black or dark brown, and the blood platelets and the nuclei of the leukocytes a reddish purple.

7. *Great care should be exercised to avoid mistaking the blood platelets accidentally superimposed upon red cells for malarial parasites. These platelets are frequently surrounded by an unstained halo. Precipitated stain, dirt, bacteria, etc., may constitute other sources of error.*

8. Precipitated stain granules may be removed by immersing the slide for a second or two in 95 per cent alcohol and immediately washing with distilled water.

**Thick Stained Film Method.**—This method is useful when there are but few parasites and thin films are negative. It is of particular value for the detection of plasmodia in malarial surveys and in patients with chronic malaria or under treatment.

1. It is essential to carefully clean the skin with alcohol and gauze in order that the blood be free of dirt, bacteria, or other débris. The slides should be perfectly clean.

2. Place 4 medium-sized drops of blood on a slide at the corners of a  $\frac{1}{2}$  inch square.

3. Draw the drops into a pool with a needle so that a thick moist layer  $\frac{1}{2}$  inch square is formed.

4. Allow the film to dry for  $1\frac{1}{2}$  hours in an incubator at  $37^{\circ}$  C. or overnight at room temperature, protected from dust by an inverted Petri dish. The smears should be dried only long enough to make them adhere, since too much drying will prevent satisfactory staining of the parasites.

5. Flood the film with a mixture of 4 parts of a 2.5 per cent aqueous solution of glacial acetic acid and 1 part of an aqueous solution of 2 per cent tartaric acid until a grayish white color denotes completion of dehemoglobinization. Treatment with distilled water or weak acids will also produce dehemoglobinization.

6. Wash with water, allow to dry and stain with Wright's stain as in the staining of blood films for differential leukocyte counts.

**Thick Stained Film Method of Barber and Komp.**—In this method preliminary dehemoglobinization and fixation are not required:

1. Prepare thick film of blood as described above. After the film has dried enough to make it adhere to the slide proceed with staining.

2. For this purpose dilute 1 part of Giemsa's stain with 6 parts of neutral or slightly alkaline distilled water (pH 7.0 to 7.2).

3. Place slide in the diluted stain for about 30 minutes.

4. Place slide in distilled water for about 5 minutes for partial decolorization. Examine under the microscope. If the smear shows a deep blue background and the leukocytes are almost black, it is overstained and probably useless, although longer washing in distilled water may decolorize it somewhat. The time required for staining depends upon the dilution of the stain, the volume used, and the thickness of the smear. Pampana has advised diluting the stain with 20 parts of a buffer solution of pH 7.2.

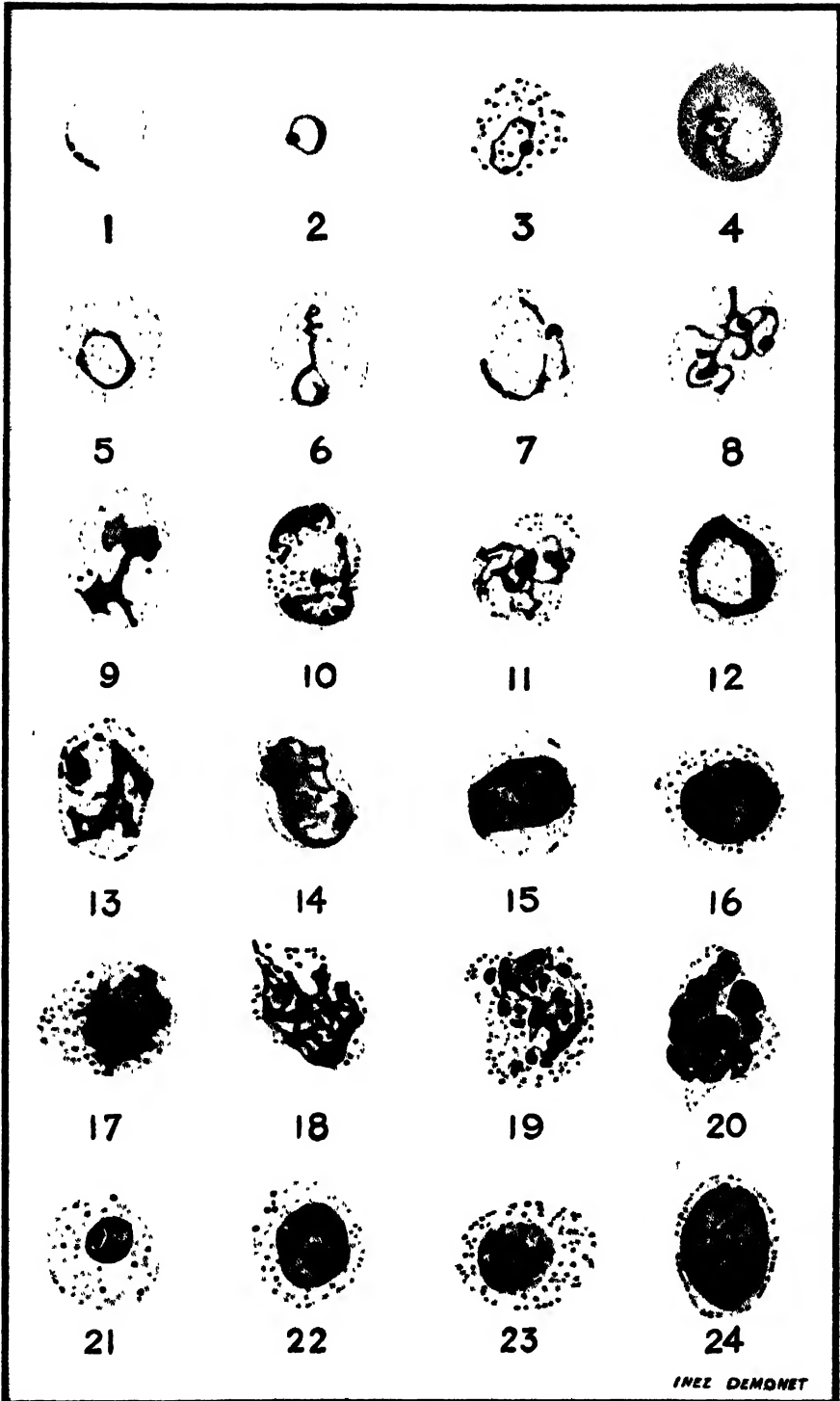
5. Drain and allow to dry in the air. Examine with oil immersion objective.

**PLATE XII.—*Plasmodium vivax***

1. Normal sized red cell with marginal ring form trophozoite.
2. Young signet ring form trophozoite in a macrocyte.
3. Slightly older ring form trophozoite in red cell showing basophilic stippling.
4. Polychromatophilic red cell containing young tertian parasite with pseudopodia.
5. Ring form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schüffner's stippling. This stippling does not appear in all cells containing the growing and older forms of *P. vivax*, but it can be found with any stage from the fairly young ring form onward.
- 6, 7. Very tenuous medium trophozoite forms.
8. Three ameboid trophozoites with fused cytoplasm.
- 9, 11, 12, 13. Older ameboid trophozoites in process of development.
10. Two ameboid trophozoites in one cell.
14. Mature trophozoite.
15. Mature trophozoite with chromatin apparently in process of division.
- 16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").
20. Mature schizont.
- 21, 22. Developing gametocytes.
23. Mature microgametocyte.
24. Mature macrogametocyte.

(From *Manual for the Microscopical Diagnosis of Malaria in Man* by Aimee Wilcox, Bulletin No. 180, National Institute of Health, 1942.)

PLATE XII



*See description of this plate on opposite page.*





6. Wright's stain may be employed in the same manner, using a 1:30 dilution with distilled water, but is not as satisfactory as Giemsa's stain.

**Concentration Method of Bass and Johns.**—1. Draw 10 cc. of blood from a vein and place in a tube carrying 0.2 cc. of the following solution:

Sodium citrate .....	5 gm.
Dextrose .....	5 gm.
Water (distilled) .....	10 cc.
Dissolve with aid of heat.	

2. Divide the blood between 2 centrifuge tubes and centrifuge at high speed (2500 revolutions per minute) for the proper length of time (about 5 minutes for a centrifuge with a radius of 18 centimeters).

3. With a capillary pipet remove the supernatant plasma. Then carefully skim off the grayish layer of leukocytes and parasites and place in a tube about 12 centimeters long with an inside diameter of about 0.5 centimeter (made from ordinary glass tubing). Add an equal volume of plasma.

4. Mix and centrifuge as before.

5. With a capillary pipet draw off the "cream." Mix by forcing in and out upon a slide. Then draw into the pipet and seal the tip in a flame. Nick with a file and break off above the blood column.

6. Place this slender tube in the centrifuge and centrifuge again as above.

7. The leukocytes will form a grayish layer upon the surface of the sediment. This and the upper portion of the erythrocyte layer contain the parasites.

8. Nick with a file and break off the capillary tube at a point 1 to 2 millimeters below the bottom of the leukocyte layer.

9. With a capillary pipet, the stem of which will pass inside the capillary tube, remove the small amount of red cells and leukocytes together with a little plasma.

10. Mix well, make smears on slides, and stain with Wright's stain in the usual way.

11. Best results are obtained with estivo-autumnal crescents and adult tertian and quartan parasites. Very young parasites do not concentrate as well, if at all.

**Splenic Puncture Method.**—On account of its dangers this method should be employed only in chronic malaria after other methods have failed. Prolonged coagulation time of the blood is a contraindication.

1. Have patient flat on the back with hands folded beneath the head. Children may be given a general anesthetic.

2. Sterilize area of puncture with tincture of iodine.

3. Have an assistant hold the spleen firmly against the diaphragm and costal region.

4. Using a sterile 15-gauge needle and a dry syringe, puncture the spleen with a direct firm thrust, while the patient holds the breath.

5. Without delay make forcible aspiration and then immediately withdraw the needle sharply in one motion.

6. Have patient remain incumbent for  $\frac{1}{2}$  to 2 hours, and check the pulse rate at intervals for signs of hemorrhage. An abdominal binder may be advisable.

7. Eject 2 drops of the aspirated material onto slides and spread in the same manner as in the thin blood film method.

**PLATE XIII.—*Plasmodium malariae***

1. Young ring form trophozoite of quartan malaria.
- 2, 3, 4. Young trophozoite forms of the parasite showing gradual increase of chromatin and cytoplasm.
5. Developing ring form trophozoite showing pigment granule.
6. Early band form trophozoite—elongated chromatin, some pigment apparent.
- 7, 8, 9, 10, 11, 12. Some forms which the developing trophozoite of quartan may take.
- 13, 14. Mature trophozoites—one a band form.
- 15, 16, 17, 18, 19. Phases in the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
21. Immature microgametocyte.
22. Immature macrogametocyte.
23. Mature microgametocyte.
24. Mature macrogametocyte.

(From *Manual for the Microscopical Diagnosis of Malaria in Man* by Aimee Wilcox, Bulletin No. 180, National Institute of Health, 1942.)

PLATE XIII . .



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INEZ DEMORET

*See description of this plate on opposite page.*



8. Fix, stain and examine as in the thin blood film method.

**Culture Method of Bass and Johns.**—This method is usually successful in cultivating 3 or 4 generations of the malarial plasmodia. Its chief use is as a control on treatment, since positive cultures may be obtained when blood films are negative.

1. Place 10 cc. of blood secured aseptically by venipuncture in a test tube containing 0.1 cc. of a 50 per cent solution of glucose.

2. Defibrinate the blood with a sterile wire or glass rod.

3. Transfer the defibrinated blood to several narrow sterile test tubes to a depth of about 2.5 cm. and centrifuge until there is about 1.2 cm. of serum above the cells.

4. Incubate at 37 to 39° C. The parasites develop in the thin upper layer of cells just below the clear serum. Those in the deeper lying cells die. To observe development, red cells from the upper layer are drawn up with a capillary pipet. Thin wet or thin stained films are prepared and examined. Should more than one generation be desired the buffy layer of leukocytes must be carefully removed and the erythrocytes placed in fresh serum. Only the parasites in the erythrocytes escape phagocytosis. Sinton, following the same general method (*Indian J. M. Res.*, 10: 203, 1922), has developed a more refined but more complicated technic, using sealed capillary pipets incubated at 35 to 38° C.

**Species Identification of Plasmodia.**—*Plasmodium vivax* (tertian malaria).—The erythrocytes are larger than normal and palely stained (Plate XII).

The young parasite is about  $\frac{1}{3}$  of the diameter of the infected cell. It somewhat resembles a signet ring, the chromatin mass (staining red) representing the signet or stone; the peripheral (blue staining) cytoplasm, the band; with a clear unstained vacuole forming the center.

The growing parasite or trophozoite is irregular in shape and may show vacuoles, one or more chromatin masses, and even scattered pigment. The red cell may show Schuffner's dots, which are tiny and pale pink in color.

The segmenting parasite, the schizont, fills the cell. It shows 15 to 20 chromatin masses, each surrounded by a mass of blue cytoplasm (merozoite). They are arranged irregularly. The pigment is aggregated in a mass which may be seen near the center.

The sexual forms (macro and microgametocyte) fill the cell almost completely, are round or oval, having a deep blue cytoplasm, large mass of chromatin and considerable pigment.

In tertian malaria, any of the above forms may be found in a single smear of the peripheral blood, although one of the forms will probably predominate.

*Plasmodium malariae* (quartan malaria). In quartan malaria, the red cells are normal in size and color (Plate XIII).

The young parasite or ring is composed of a large chromatin mass, a central unstained vacuole, and a heavy rim of blue-staining protoplasm. The diameter of the young trophozoite is about  $\frac{1}{3}$  of the diameter of the red cell.

The growing parasite is elongated or band-like (band forms) made up of blue cytoplasm with irregular and elongated red-staining chromatin masses. The pigment is coarse and scattered throughout. The diseased red cells do not show Schuffner's dots.

The mature parasite, or schizont, practically fills the cell, showing 6 to 10 chromatin masses, each surrounded with a blue cytoplasm. Their arrangement resembles a rosette. Coarse pigment is centrally located.

The sexual forms are similar to those of *P. vivax*. The pigment, however, is coarser.

**PLATE XIV.—*Plasmodium falciparum***

1. Very young ring form trophozoite.
2. Double infection of single cell with young trophozoites, one a "marginal form", the other "signet ring" form.
- 3, 4. Young trophozoites showing double chromatin dots.
- 5, 6, 7. Developing trophozoite forms.
8. Three medium trophozoites in one cell.
9. Trophozoite showing pigment, in a cell containing Maurer's spots.
- 10, 11. Two trophozoites in each of two cells, showing variation of forms which parasites may assume.
12. Almost mature trophozoite showing haze of pigment throughout cytoplasm. Maurer's spots in the cell.
13. Aestivo-autumnal "slender forms".
14. Mature trophozoite, showing clumped pigment.
15. Parasite in the process of initial chromatin division.
- 16, 17, 18, 19. Various phases of the development of the schizont ("presegmenting schizonts").
20. Mature schizont.
- 21, 22, 23, 24. Successive forms in the development of the gametocyte—usually not found in the peripheral circulation.
25. Immature macrogametocyte.
26. Mature macrogametocyte.
27. Immature microgametocyte.
28. Mature microgametocyte.

(From *Manual for the Microscopical Diagnosis of Malaria in Man* by Aimee Wilcox, Bulletin No. 180, National Institute of Health, 1942.)

# PLATE XIV



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INEZ DEMONET

See description of this plate on opposite page.





In quartan malaria any or all of the above forms may be present in a single smear of the peripheral blood.

*Plasmodium falciparum* (estivo-autumnal malaria).—The diseased red cells in this infestation are smaller than normal and may be distorted (Plate XIV).

The young parasites or ring forms are very delicate, with a small chromatin dot, a central vacuole, and a delicate blue-staining peripheral ring-like cytoplasm. It is common for two or more parasites to be seen in the same cell. (This is rarely or never seen in either *P. vivax* or *P. malariae*.) Some of the rings may have more than one chromatin dot.

The growing parasite or trophozoite and the mature parasite or schizont are only found in the peripheral blood in overwhelming infections.

The sexual forms are very characteristic. The female (macrogametocyte) is crescent-shaped, pale blue in color with the ends slightly pointed. A large mass of chromatin and pigment is compactly arranged near the center. The red cell is deformed and very pale. The male (microgametocyte) is crescent or sausage shaped, of a blue gray color, with considerable chromatin, and pigment near the center arranged more diffusely.

In estivo-autumnal malaria smears of the peripheral blood will show only *the rings* and *the crescents*.

**Henry's Melano-Flocculation Test.**—This test depends upon changes in the serum proteins, especially an increase of euglobulin. It gives a high percentage of positive reactions in malaria, but at times is positive in syphilis and other diseases. The technic is as follows:

1. Prepare an antigen from the choroidal melanin of the ox-eye in distilled water containing 0.005 per cent formaldehyde and of such opacity that a 1:10 dilution will correspond to an optical density of 48 to 49 photometric degrees.

2. Mix 1 part of serum with 4 parts of antigen in a stoppered test tube and incubate at 37° C. for 3 hours.

3. Allow the tube to stand at room temperature for 30 minutes.

4. Distinct flocculation of the melanin indicates a positive reaction, provided the serum and antigen controls are clear.

5. Because of the difficulty of reading weakly positive reactions the use of a photometer for estimating the optical density is advised. Under these circumstances 1 to 12 degrees is regarded as negative, 13 to 18 doubtful, 19 to 100 positive for malaria.

Otherwise the tyrosin colorimetric test of Proske and Watson (*U.S. Pub. Health Ref.*, 54: 158, 1939) to measure euglobulin may be employed. It is based on the fact that proteins possess a chromogenic property which can be measured quantitatively against the color produced by pure tyrosin in the presence of a phenol reagent.

## METHODS OF EXAMINATION FOR MICROFILARIAE

**Principles.**—1. Filariasis is usually due to infestation of the lymphatics by *Wuchereria bancrofti* (*Filaria bancrofti*), *Wuchereria malayi*, *Acanthocheiloma perstans* or *Mansonella ozzardi* (*Filaria perstans*).

2. In infestments due to *W. bancrofti* and *W. malayi* laboratory diagnosis is based upon finding the microfilariae during the night and especially between 10 P.M. and

2 A.M. In *A. perstans* and *M. ozzardi* infestments the microfilariae occurring in the blood are nonperiodic.

3. *Onchocerciasis* is due to infestation with *Onchocerca volvulus*. Laboratory diagnosis is based upon the finding of microfilariae or dead worms in material aspirated from suspected tumors or ocular lesions, or of macerated tissue removed by biopsy.

4. *Loasis* is due to infestation with *Loa loa* (*Filaria loa*, *Filaria oculi*). Laboratory diagnosis is based upon finding microfilariae in the blood during the day or by the recovery of worms from their tunnels.

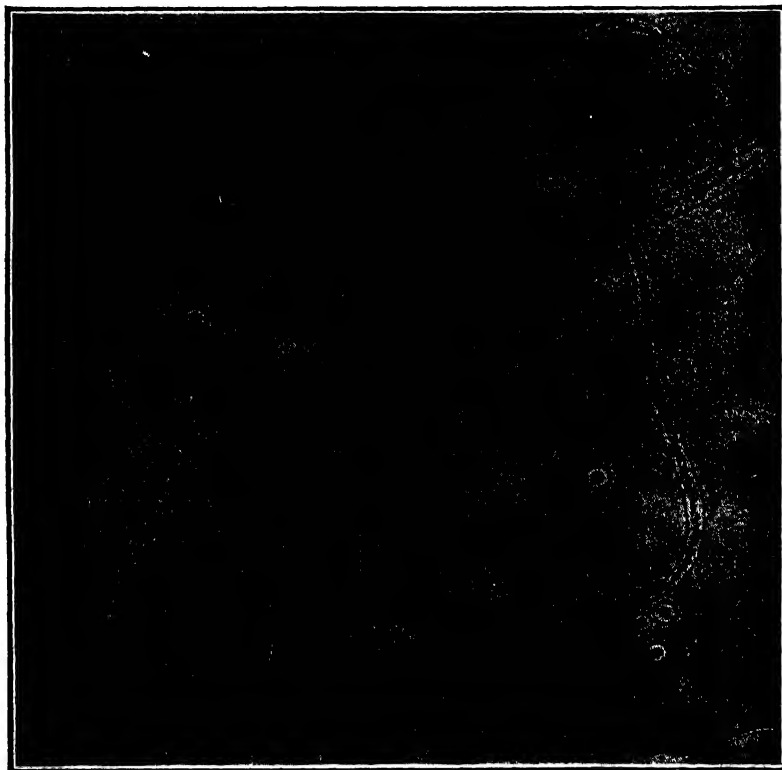


FIG. 273.—LARVAE OF *FILARIA BANCROFTI*

(After Railliet. From Braun, *Die thierischen Parasiten des Menschen*, Bale Sons and Danielsson, London.)

**Blood Examinations.**—1. In the laboratory diagnosis of filariasis due to *Wuchereria bancrofti* (*Filaria bancrofti*), *Wuchereria malayi*, *Acanthocheiloma perstans* and *Mansonella ozzardi*, the methods of examination for microfilariae in the blood may be by means of fresh wet films, thin stained films and thick stained films as previously described in the diagnosis of malaria. Fresh wet films are preferred. Preparations may be examined by the low power objective. Since microfilaria are very transparent subdued light should be used.

2. Concentration methods, however, are frequently required. A simple method consists of collecting 1 cc. of blood from a finger or ear in 5 cc. of a 2 per cent

solution of glacial acetic acid, mixing thoroughly and centrifuging. Wet and stained smears of the sediment are examined for microfilaria.

The method of Stubbs and Live (*Jour. Amer. Vet. Med. Assoc.*, 40: 680, 1935; *ibid.* 45: 686, 1938) is highly recommended as follows: (a) Collect 5 to 10 cc. of blood by venipuncture and place in a test tube; (b) allow blood to clot, separate the clot from the wall of the tube and allow to stand for clot retraction (usually 3 to 4 hours); (c) carefully separate the serum and place in a test tube; (d) add two volumes of 5 per cent acetic acid or two volumes of 2 per cent hydrochloric acid; (e) invert the tube several times and centrifuge at 600 to 1000 r.p.m. for 6 minutes or allow to stand overnight; (f) remove the supernatant fluid; (g) mix the sediment, place a drop on a slide, cover with coverglass and examine with the low power objective.

**Aids for the Species Identification of the Microfilaria.**—The microfilaria are easily detected in the peripheral blood due to their large size, and their intentionless lashing movement, which agitates the red cells and immediately attracts the eye when specimens are examined under the low power of the microscope.

*Wuchereria bancrofti*.—The *W. bancrofti* is a sheathed embryo. It measures 300 by 7.5 micra. It forms graceful curves, is regular in outline, and is rarely angulated. The head is the same width as the body. The body matrix, which is made up of numerous nuclei, does not extend to the extreme limits of the tail, which is pointed. The embryos have a nocturnal periodicity. The blood should be examined from between 9 P.M. and 2 A.M. (Fig. 273).

*Microfilaria Loa*.—*M. loa* measures 250 by 7 micra. It is a sheathed embryo, the body matrix cells extending to the extreme limits of the tail, and ends squarely at the head. Its outline is slightly irregular, the head slightly flattened and broader than the body. Its curves are not graceful. It has a diurnal periodicity. The blood should be examined at mid-day.

*Acanthocheiloma perstans*.—This embryo is unsheathed, that is, the body cavity containing the nucleus is not covered by a surrounding layer or sheath. The head is blunt, the organism measures 200 by 5 micra. This embryo does not exhibit periodicity, and is usually found in the peripheral blood at all times.

*Dirofilaria immitis*.—Adult filaria are found most often in the right heart and pulmonary artery of the dog. They are long white worms 12 to 30 cm. in length and 0.7 to 1.3 cm. in width. Sexually mature females discharge large numbers of larvae into the circulating blood. The larvae are 280 micra in length and about the width of a red cell. They are very active and do not exhibit periodicity.

## METHODS OF EXAMINATION FOR DRACUNCULUS MEDINENSIS

Dracontiasis is due to infestation with the Guinea worm *Dracunculus medinensis*. Laboratory diagnosis is based upon finding the female worms in the subcutaneous tunnels of papulovesicular lesions of the feet or elsewhere or of larvae in the vesicular fluids. Dead or calcified worms may be located by roentgen-ray examination. The disease is common in Asia, Africa, South America and the West Indies.

The gravid females measure 500 to 1,200 mm. in length and 0.9 to 1.7 mm. in diameter, averaging about 600 mm. The anterior end bears a cuticular shield and is

pairs of papillae. The larvae on liberation from the female have a slender rhabditiform shape with a long, filiform tail tapering to a sharply pointed tip. They reach maximal development after 21 days. They are actively motile in the body during the first 4 or 5 weeks after which they become inactive and tightly coiled. If the worm is broken during extraction and the larvae escape into the subcutaneous tissues, a severe inflammatory reaction with fever ensues with possible secondary bacterial infection.

### METHODS OF EXAMINATION FOR *TRICHINELLA SPIRALIS*

*Trichinosis* is due to infestation with *Trichinella spiralis* from the eating of raw or improperly cooked lean pork or bear tenderloin carrying the cysts.

In the intestine the adults mature. The female is 3 to 4 mm. in length, the male is smaller. In the intestine copulation takes place and the female discharges the embryos. Some enter the lymph spaces and are eventually carried to all organs of the body by the blood, those reaching the muscles only seeming to survive.

Here they become encysted, and can be seen with the naked eye as tiny white specks in the muscle. The cysts are lemon-shaped, measuring 400 by 250 micra, and are interposed between muscle fibers. The coiled embryo is surrounded by a capsule which it secretes. A reactionary round-celled exudate, with the formation of some fibrous tissue, may form an additional surrounding layer. After a time the cysts become calcified.

1. Diagnosis may be made during the early period of diarrhea when the adult parasites are occasionally found in the feces. Also by sometimes finding the larvae in the blood between the sixth and twenty-second days after the onset of symptoms by the following method: (a) Obtain 10 cc. of blood by venipuncture; (b) mix thoroughly with 25 cc. of 2 per cent acetic acid; (c) centrifuge thoroughly; (d) examine the sediment either by placing a drop on a slide and covering with a coverglass or by smears stained with Wright's stain. The larvae are easily recognized with positive results in about 50 per cent of cases. They are also sometimes found in the spinal fluid after thorough centrifuging and preparing smears of sediment.

2. Diagnosis is usually made, however, by finding the encysted embryos in the muscles (Fig. 257) by biopsy examinations during or after the third or fourth week of the infestation. For this purpose a small piece of the muscle may be removed from the insertion of the deltoid, the head of the gastrocnemius, pectoralis major, or from the lower portion of the biceps, and should be divided into 2 parts. The first may be examined in the fresh state by taking a small fragment (about 1 mm. in thickness) and pressing out between 2 small pieces of glass or micro slide so as to make the specimen translucent (preferably a Trichine press). With a low power lens or microscope the embryo can easily be seen. The rest of the tissue should be run through as a routine tissue biopsy and stained with hematoxylin and eosin. These muscle sections will likewise reveal the encysted embryos.

**METHODS OF EXAMINATION FOR TRYPANOSOMES**

**Principles.**—1. African trypanosomiasis or “sleeping sickness” is due to infection with *Trypanosoma gambiensi* or *Trypanosoma rhodesiensi*; American trypanosomiasis or Chagas’ disease is due to infection with *Trypanosoma cruzi*.

2. Early diagnosis and treatment of African trypanosomiasis is important. Laboratory diagnosis is based upon finding the trypanosomes in the blood (especially during febrile periods) and by animal inoculation tests. Diagnosis in the late stages is based upon finding the trypanosomes in the spinal fluid and by animal inoculation tests with blood, spinal fluid or material removed from enlarged lymphatic glands by puncture. Cultures are not usually successful.

3. The laboratory diagnosis of Chagas’ disease is difficult. Trypanosomes may be found in the blood during acute febrile periods in children or adults; animal inoculation tests and “xenodiagnosis” are helpful.

**Blood Examinations.**—1. These may be conducted with fresh wet films, thin stained films or thick stained films in the same manner as in examinations for the plasmodia of malaria. Trypanosomes, however, are never found within cells but are always free in the plasma (Fig. 274).

2. A concentration method may be conducted as follows: (a) Collect 5 to 10 cc. of blood by venipuncture and place in a test tube carrying 1 cc. of 1 per cent sodium citrate in saline solution to prevent coagulation; (b) filter through cheesecloth into a centrifuge tube; (c) centrifuge for 20 minutes and remove the supernatant fluid; (d) hemolyze the erythrocytes by adding distilled water or 2 per cent acetic acid; (e) centrifuge for 30 minutes and discard the supernatant fluid; (f) prepare wet and stained smears of the sediment and examine. Otherwise 10 cc. of citrated blood may be thoroughly centrifuged, the plasma removed and smears prepared of the leukocyte cream layer, which is just above the packed erythrocytes.

**Spinal Fluid Examination.**—1. Conduct spinal puncture and collect about 10 cc. of fluid.

2. Centrifuge at high speed for 15 minutes.

3. Remove the supernatant fluid and prepare smears of the sediment; stain with Wright’s stain and examine.

4. These examinations are required in the late stages of “sleeping sickness” since trypanosomes are not likely to be found in the blood.

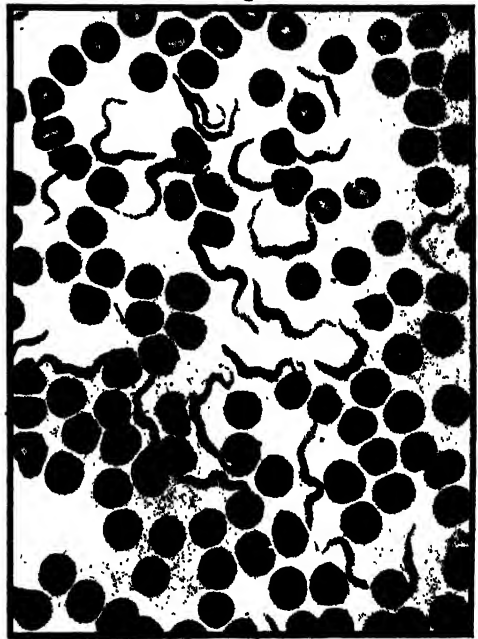


FIG. 274.—BLOOD OF RAT WITH TRYPANOSOMIASIS

Film prepared four days after intraperitoneal inoculation with *Trypanosoma equiperdum*. (From Kolmer, *Chemotherapy*, W. B. Saunders Co., Philadelphia.)

**Lymph Gland Examinations.**—Lymph glands may be examined as follows when blood examinations are negative: (a) Use a large sterile needle and a dry syringe or one containing a few drops of sterile saline solution, which may be injected into the gland before aspiration; (b) prepare the skin over an enlarged gland, hold the gland firmly in position and thrust the needle sharply into its substance; (c) aspirate and remove the needle; (d) prepare wet and stained smears of aspirated material and examine for trypanosomes.

**Animal Inoculation.**—1. Inoculate white rats or mice intraperitoneally with 1 cc. of citrated blood, 1 to 2 cc. of spinal fluid or material aspirated from lymphatic glands. Guinea-pigs and rabbits may be used, but are less satisfactory because much less susceptible to infection. For *T. gambiensi* monkeys are preferred but cannot generally be employed.

2. Make daily examinations of the blood for trypanosomes using fresh wet and stained preparations. In the case of rats and mice blood may be secured by snipping off the tips of the tails. In positive cases the trypanosomes usually appear between the third and fourteenth days after inoculation and remain in the blood quite constantly until death occurs.

**Xenodiagnosis (Brumpt).**—This method is used only as an aid in the diagnosis of Chagas' disease. It consists of allowing laboratory bred triatomids to bite the individual suspected of having the disease. If infection is present, the trypanosomes multiply rapidly in the intestine of the bug and an examination of the intestinal contents will result in their detection. This method is sometimes successful when the trypanosomes are so few in number in the blood that they cannot be found microscopically.

**The Species Identification of the Trypanosomes of Man.**—The trypanosomes are the most highly developed of the hemoflagellates. For species identification the worker must be familiar with the structural details which are described in the paragraphs below.

The parasites have a fusiform body with pointed ends. They are flattened from side to side. In the central part is a large nucleus. In the posterior end a small chromatic mass is found called the "parabasal body." Immediately adjacent is a blepharoplast, from which an undulating membrane and marginal flagellum arise. The flagellum in most species, after forming the edge of the undulating membrane, extends beyond the anterior end as a free process of varying length. In Figure 275 these structures have been represented diagrammatically.

***Trypanosoma Gambiense.***—It is 15 to 30 micra in length, and produces a chronic form of sleeping sickness in man. It has a large nucleus centrally placed, a small parabasal body and blepharoplast located at the posterior end. The undulating membrane is considerably convoluted and wide. In the peripheral blood the forms may be long and slender or short and plump. In the former the flagellum is long, in the latter it may be absent. Direct stained smears of the peripheral blood may be negative owing to the relatively small number of organisms present.

When blood or emulsified glandular tissue is injected into a white rat, the animal develops a rather chronic form of the disease, although the parasites will be found in the blood. Stained smears reveal forms similar in structure with those found in man.

***Trypanosoma Rhodesiense.***—It measures 15 by 30 micra. This organism produces

a rapidly fatal variety of sleeping sickness. Its morphology is similar to that of *T. gambiense*. Only a few parasites are found in the peripheral blood, so that direct thin blood smears may be negative.

When blood or gland tissue is inoculated into animals, a rapidly fatal trypanosomiasis is produced. Examination of stained smears of the animals' blood shows forms similar to those described under *T. gambiense*, and in addition forms showing the "posterior nucleus." In these, the nucleus is in the posterior end, closely associated with the parabasal body and blepharoplast sometimes being even posterior to these structures. These posterior nucleated forms serve to differentiate the two species.

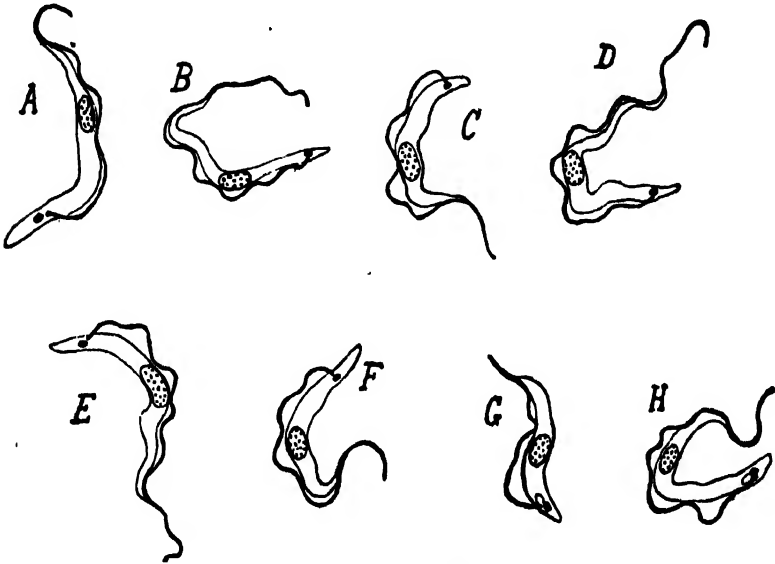


FIG. 275.—THE MOST IMPORTANT TRYPANOSOMES PARASITIC IN VERTEBRATES

A, *Tr. lewisi*. B, *Tr. evansi* (India). C, *Tr. evansi* (Mauritius). D, *Tr. brucei*. E, *Tr. equiperdum*. F, *Tr. equium*. G, *Tr. dimorphon*. H, *Tr. gambiense*.  $\times 1500$ . (After MacNeal.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

*Trypanosoma Cruzi*.—This parasite produces a disease in Central and South America called "Chagas' disease." The organism is 20 micra long, showing both slender, long and short, plump forms. The nucleus is centrally placed. An important differential structure is the large oval posteriorly-placed parabasal body. The undulating membrane is narrow and very slightly convoluted. The mature trypanosome forms of *T. cruzi* are only occasionally seen in direct thin blood smears. The parasite forms micro cyst-like colonies within various organs, particularly the muscles, including the heart. In these, various developmental forms are seen, with the Leishmania bodies predominating. Upon the rupture of such cysts the mature forms appear in the peripheral blood.

The organisms may be found in the peripheral blood during the acute phases of the disease, or the organisms may be recovered from the peripheral blood or tissues of animals.



**METHODS OF EXAMINATION FOR LEISHMANIA**

**Principles.**—1. Kala-azar or visceral leishmaniasis is due to infection with *Leishmania donovani*. Laboratory diagnosis is based upon (a) finding leishmania in the neutrophils or large monocytes of the peripheral blood; (b) the examination of material removed from the liver, spleen or lymphatic glands by puncture; (c) cultures of the spleen and (d) by inoculation of hamsters with blood or puncture material.

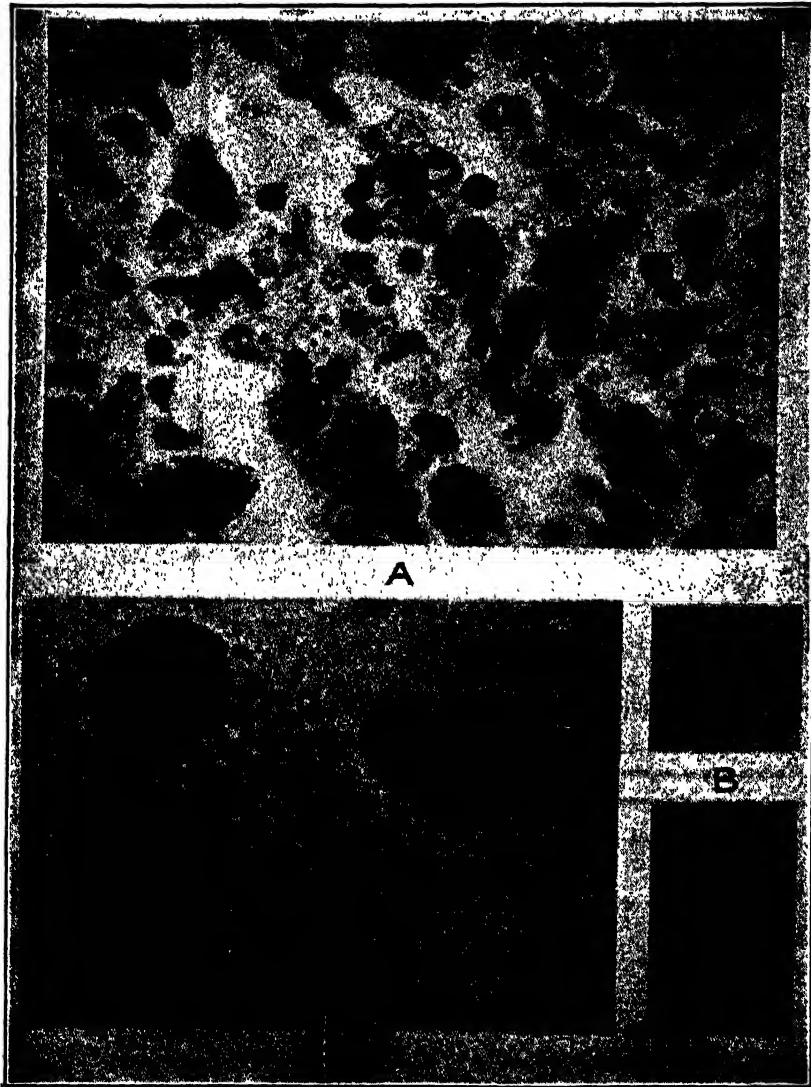


FIG. 276.—*LEISHMANIA TROPICA*

A, Section of cutaneous lesion showing organisms included in large mononuclear cells; C, a large mononuclear cell showing cytoplasm filled with organisms in film stained with Wright's stain; B and D, dividing forms, Wright's stain. (After Wright.) (From Zinsser and Bayne-Jones, *Textbook of Bacteriology*, 7th Edition, D. Appleton-Century Co., New York.)

2. *Leishmania tropica* or oriental sore is due to infection with *Leishmania tropica*. Laboratory diagnosis is based upon (a) finding leishmania in material from ulcers, especially by puncture of the margins and (b) by cultures.

3. *Leishmania braziliensis* or espundia is due to infection with *Leishmania braziliensis*. Laboratory diagnosis is based upon (a) finding leishmania in material obtained by puncture of the margins of initial lesions or from secondary nodules or ulcers or (b) by cultures.

4. Supplementary examinations of diagnostic value consist of (a) Napier's aldehyde test, (b) Chopra's antimony test and (c) Sia's precipitative test.

**Blood Examinations.**—In suspected kala-azar blood examinations consist in the preparation of thin and thick (preferred) blood films stained with the Wright or Giemsa stains in exactly the same manner as previously described for the detection of the malarial plasmodia. The large monocytes and neutrophils are examined for intracellular *L. donovani*.

**Tissue Examinations.**—1. In *Leishmania tropica* (oriental sore) and *Leishmania braziliensis* (espundia) the lesions are superficially cleaned with alcohol and allowed to dry. If possible, the serous exudate from a lesion is obtained by puncturing the indurated margin with a sterile capillary pipet or large needle. Another method is to scrape the surface of the lesion with a scalpel until it bleeds. The blood is removed after the hemorrhage has ceased and the serous exudate which collects is then taken for examination. Ordinarily, the removal of material by scraping with a scalpel is sufficient. Smears of the material are prepared on slides and stained with the Wright or Giemsa stains. The organisms are to be found within the reticulo-endothelial cells (Fig. 276).

2. In kala-azar material may be secured by aspiration of the spleen, lymphatic glands or liver. This material may be examined by the preparation of smears stained by Wright's or Giemsa's stains and by cultures. Leishmanian parasites are usually less abundant in the liver than in the spleen. Liver puncture may be conducted as follows: (a) Exclude patients with leukemia, hemorrhagic disease, malaria, and hydatid cyst; (b) prepare the skin and administer a local anesthetic; (c) insert a dry sterile needle, not more than  $3\frac{1}{2}$  inches long, into the eighth intercostal space in the anterior axillary line 1 to  $1\frac{1}{2}$  inches from the costal margin, directing it inward, upward and backward (several punctures may be made in different parts through the same aperture); (d) aspirate material and withdraw the needle. Apply a dressing. With due care aspiration is a safe procedure.

**Cultural Examinations.**—A satisfactory medium for the cultivation of the Leishmania is that of Novy, MacNeal and Nicolle (N.N.N.). The technic may be as follows:

1. Place 0.25 to 0.5 cc. of blood in 20 cc. of 1.5 per cent sodium citrate in physiological salt solution.

2. Shake the mixture gently, and place in the ice-box over night.

3. Centrifuge and decant the supernatant fluid. Transfer the corpuscles with a sterile pipet into the water of condensation of a tube of N.N.N. medium.

4. Incubate at 22 to 25° C.

5. Examine this fluid from time to time by staining smears for the presence of the herpctomonas forms of the parasite.

Only a few organisms are found in the peripheral blood, and therefore the growth

may be slow. Cultures should not be considered negative until after 3 or 4 weeks. Ordinarily, however, they should show parasites in from 3 to 14 days (Fig. 277).

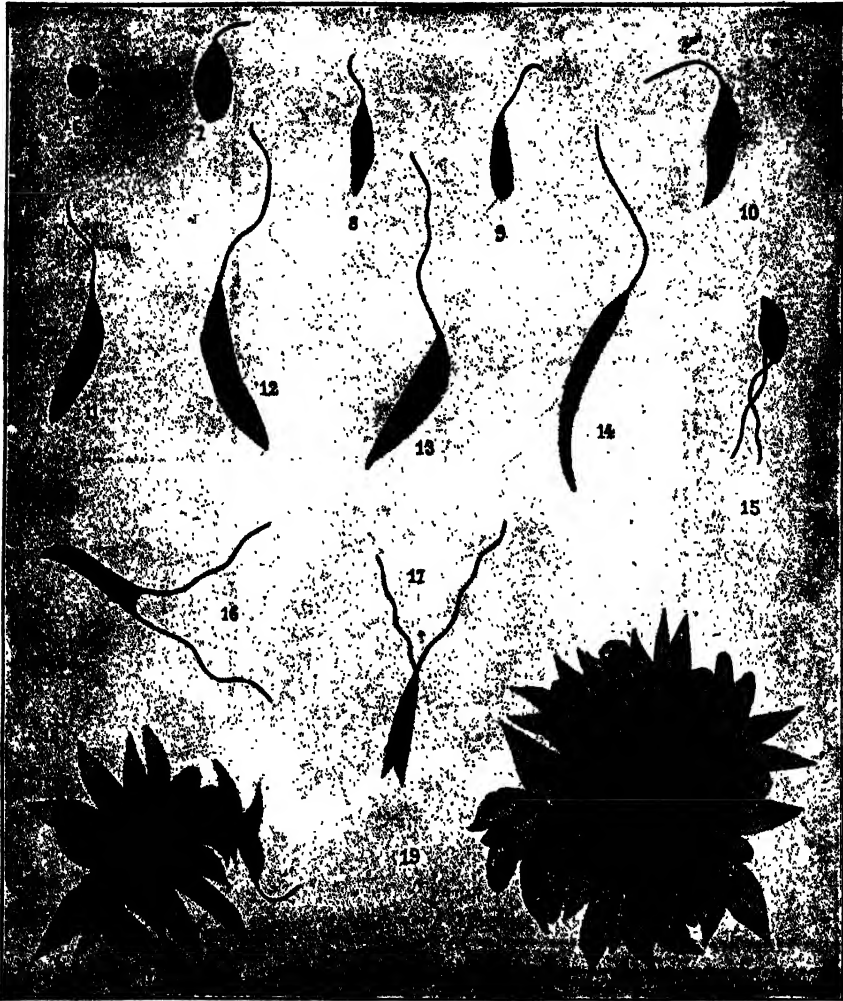


FIG. 277.—CULTURAL FORMS OF LEISHMAN-DONOVAN BODIES

(From Park, Williams and Krumwiede, *Pathogenic Microorganisms*, Lea and Febiger, Philadelphia.)

6. Aspirated material from spleen or liver may be inoculated directly into the water of condensation of tubes of N.N.N. media and incubated at 22 to 25° C. as described above.

7. Material from lesions in cutaneous Leishmaniasis or lesions about the mucous membranes in espundia can likewise be inoculated directly.

8. Great care should be taken to avoid bacterial contamination. Wherever possible, lesions which have not yet undergone ulceration should be aspirated with a sterile syringe, thus minimizing the chances of bacterial contamination.

**Aids to the Identification of Leishmania.**—The diagnosis of Leishmaniasis usually depends upon finding the so-called "Leishman-Donovan" bodies (Leishmania forms). These are 2 to 4 micra in diameter, are usually oval or rounded in shape, and are found within the large reticulo-endothelial cells of the tissue or monocytes of the blood. They are said to somewhat resemble a cockle shell, because of the large eccentrically placed chromatin bodies. The larger or nucleus is round or oval, in front of which is a rod-shaped deep-staining body, the parabasal body and basal granule.

Stained with Leishman's or Wright's stain, the protoplasm is a pale blue, the nucleus stains dark, the parabasal body still darker.

In culture, the organisms develop heptomad forms 14 to 20 micra in length. Some are fusiform in shape, with a centrally-placed oval nucleus and parabasal body at the anterior end, from which extend anteriorly the varying-length flagellum. No undulating membrane is present.

**Napier's Aldehyde Test.**—The aldehyde or formal-gel test of Napier has proved of diagnostic value in kala-azar, from 82 to 85 per cent of patients with active infection of 4 months' duration giving a positive reaction. This test, however, is not specific since somewhat similar reactions may also occur in tuberculosis, leprosy, trypanosomiasis, malaria, and schistosomiasis. The technic is as follows:

1. Place 1 cc. of patient's serum in a small test tube.
2. Add 1 drop of commercial formalin (36 per cent formaldehyde), mix thoroughly, and allow to stand at room temperature.
3. In positive cases the serum immediately becomes opaque and will assume a stiff white jelly-like consistency, resembling the coagulated white of egg, in from 3 to 30 minutes.
4. If negative no reaction should occur in 24 hours.

**Chopra's Antimony Test.**—This test is stated to be more sensitive than Napier's test in early kala-azar but gives more falsely positive reactions in other diseases. The technic is as follows:

1. Place 0.2 cc. of whole serum and serum diluted 1:10 with distilled water in two small test tubes (65 x 4 mm.).
2. Carefully overlay with a 4 per cent solution of urea-stibamine in distilled water, allowing the antimony solution to run slowly along the side of the tubes.
3. If positive a thick flocculent disk will form at the junction of the two fluids within 10 to 15 minutes. Reaction may rarely be delayed for 1 to 2 hours.

**Sia's Precipitative Test.**—1. Add 20 cmm. of blood to 0.6 cc. of distilled water by a hemoglobin pipet.

2. Gently agitate the mixture.
3. Observe at 15-minute intervals up to an hour.
4. A 4-plus reaction is shown by sedimentation of a flocculent precipitate within 15 minutes, a 3-plus within 30 minutes, a 2-plus within 45 minutes and a 1-plus within an hour or longer.

### METHODS FOR IDENTIFICATION OF PROTOZOA FOUND IN THE MOUTH

**Endamoeba Gingivalis.**—This ameba has a wide geographical distribution, and is frequently found about the teeth, particularly those undergoing caries, and in tartar accumulations.

The organism is similar in morphology to the *Endamoeba histolytica*. It measures from 10 to 20 micra. It has well-marked motility. The pseudopodes are short and rather blunt. The ectoplasm is distinct. The organism ingests cell-fragments from food, and may even ingest red cells. The nucleus is much like that of the *Endamoeba histolytica*. See Figure 278.

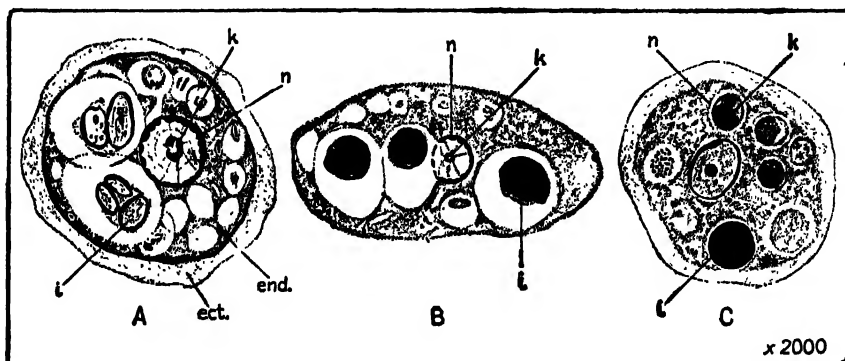


FIG. 278.—ENDAMOEBIA GINGIVALIS

A, trophozoite with a peripheral film of ectoplasm, but no pseudopodia. Food vacuoles with bacteria and with leukocytic nuclei. B, trophozoite with three large food vacuoles with leukocytic nuclei, two of which still show traces of adherent cytoplasm. Small vacuoles with remnants of food bodies but no bacteria. C, trophozoite showing ingested bodies and character of nucleus. *ect.*, ectoplasm; *end.*, endoplasm; *i*, inclusion nuclei; *k*, karyosome; *n*, nucleus (A and B redrawn from Kofoed and Swezy, 1924; C, redrawn from Goodey and Wellings, (1919). (From Belding, *Textbook of Clinical Parasitology*, D. Appleton-Century Co., New York.)

**Method of Examination.**—Scrapings from about the teeth and bits of tartar are selected, and examined for the trophozoite (motile form) by direct stained and unstained methods.

**Trichomonas Buccalis (Trichomonas Elongata).**—This flagellate is occasionally met with in material taken from about the teeth, particularly the tartar. It is practically identical in morphology with the *Trichomonas intestinalis*, and is thought by some to be the same species.

**Method of Examination.**—Material from about the gums or teeth, especially tartar, is suitable for examination, and may be examined by direct stained and unstained methods as given under Amoeba.

### METHODS FOR THE IDENTIFICATION OF PROTOZOA FOUND IN THE VAGINA

**Trichomonas Vaginalis.**—This organism is sometimes found in vaginal secretions or exudates which are acid in reaction, and may produce vaginitis. They are similar or identical in structure with the *Trichomonas intestinalis*. They measure 15 to 25 micra long and 7 to 12 micra wide.

**Method of Examination.**—The vaginal secretions or exudates are examined by direct stained or unstained methods for the motile forms.

### METHODS FOR IDENTIFICATION OF MITES

Among the important mites infesting the skin are (1) *Sarcoptes scabiei* (the itch mite); (2) *Trombicula irritans* (the harvest mite); (3) *Trombicula akamushi* (the vector of tsutsuganumushi fever); (4) *Liponyssus bacoti* (the tropical rat mite); (5) *Trombicula holosericeum* (the European harvest fly); (6) *Pediculoides ventriculosis* (the grain mite) and (7) *Glyhagus domesticus* (the sugar mite).

**Laboratory Examinations.**—A simple and rapid method for detecting mites is carried out by means of a scraping in paraffin oil by the method of Benbrook:

1. Sterilize a scalpel or other scraper in alcohol or gas flame. Cool by dipping into water. Dry.
2. Place a drop of paraffin oil in the center of a microscope slide.
3. Dip the scalpel in the paraffin oil drop (an oily scraper will pick up a specimen more easily than will a dry scraper).
4. Pinch a fold of skin showing lesions, between the thumb and forefinger, and scrape the crest of the fold with oily scalpel blade until lymph begins to ooze. Avoid drawing gross blood.
5. Transfer the scraping from the scalpel to the drop of oil on the slide.
6. Apply a coverglass to the drop with the aid of forceps.
7. Systematically examine the material under the coverglass, using the low power of the microscope and rather low illumination. The oil renders the skin scales transparent and parasites appear rather prominently. Mites may live for several days in such a preparation. In some cases, several scrapings may be necessary in order to find them.
8. Certain of the larger mites may be seen upon gross examination by scraping the lesions with a dry scalpel or knife blade and placing the scraping upon a piece of black paper or cloth exposed to sunlight and warmth. The mites may be seen as tiny white dots moving about. This method, of course, cannot be depended upon for an accurate diagnosis.

**Aids to the Species Identification of the Mites.**—*Sarcoptes scabiei*, the itch mite. This parasite produces scabies in man and mange in animals. The female *Scabiei* enters into the skin, producing burrows in which she lays from 15 to 50 eggs. These burrows measure 1 to 10 mm. in length. The usual location for the infestation is the thin skin between the fingers and toes, and about the inguinal and genital regions. The eggs measure about 14 micra in length.

In scrapings from the skin the adult female will be readily seen as a small oval mite, 400 micra in length. The male is much smaller and is similar to the female in

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shape. The head, thorax and abdomen are fused. There are 4 pair of legs. The parasite is without eyes. See Figure 279.

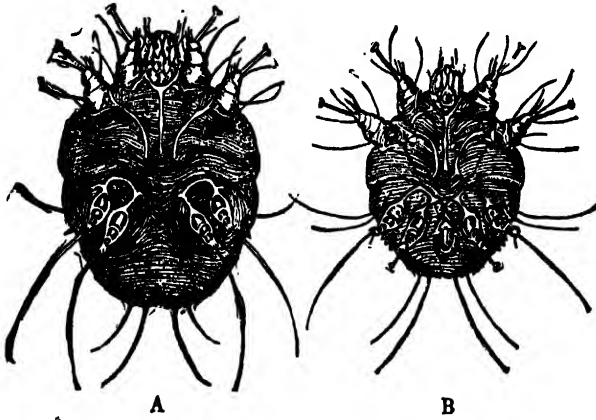


FIG. 279.—*ACARUS SCABIEI* (VENTRAL SURFACE)

A, female; B, male.  $\times 100$ . (From Schamberg, *Diseases of the Skin and Eruptive Fevers*, W. B. Saunders Co., Philadelphia.)

*Trombicula irritans*, harvest mite, red bug or chiggers. This mite produces a red itching wheal, usually with minute red spot in the center. The parasite penetrates the skin and feeds on the blood of the host. The mite is reddish, oval in shape, and has 4 pair of legs, a hairy body and pedunculated eyes. In Japan a similar species, *T. akamushi*, is important in the spread of a typhus-like disease known as "Japanese river fever," tsutsugamushi. *T. holosericeum* is the harvest mite of Europe.



FIG. 280.—FEMALE GRAIN ITCH MITE.  
 $\times 300$

(From Schamberg, *Diseases of the Skin and Eruptive Fevers*, W. B. Saunders Co., Philadelphia.)

*Liponyssus bacota* is of importance in the tropics, being partly responsible for the spread of typhus from rat to rat.

*Pediculoides ventriculosis* is associated with wheat straw, producing skin lesions and at times irregular fever among the individuals coming in contact with the infested straw. The grain or straw itch mite producing acarodermatitis urticarioides (Schamberg) is shown in Figure 280.

*Glyphagus domesticus* is the food mite, commonly found in sugar, producing in individuals

handling such infested food "grocers' itch."

## METHODS FOR IDENTIFICATION OF TICKS

There are a number of species of ticks which are pests that infest mammals, birds and men. They are primarily of importance as carriers, but the wounds produced by their bites may become secondarily infected with bacteria. They are 1 to 4 mm. in

length, flat and oval, tending to taper toward the anterior end, with no division between the cephalothorax and abdomen. They engorge themselves with blood through a heavily armatured piercing-organ, the hypostome.

**Ornithodoros Moubata.**—This is an oval, yellowish-brown tick. It is found on mammals as well as birds, and is important as a vector of West African relapsing fever in man.

**Dermacentor Andersoni, Wood Tick.**—This is reddish-brown in color, oval, 6 mm. in length. The male shows white and black markings. It is found in the western part of the United States. It infests domestic and wild animals, but is an important vector in the transmission of Rocky Mountain spotted fever and tularemia.

### METHOD FOR IDENTIFICATION OF THE TONGUE WORM

**Linguatula Serrata, the Common Tongue Worm.**—The larvae of this arachnoid is occasionally found in man. The adult female measures 10 cm. in length, and usually occurs in the nasal passages and frontal sinuses of carnivora, occasionally horses and sheep.

The adults rarely infest man. Man acquires the infestation by swallowing the ova from infested animal secretions. The ova hatch out in the intestine, and the larvae eventually reach the liver or nasal cavity and sinuses of man. The larvae are about 5 mm. in length, slightly flattened tongue shape and tapering at the end.

### METHODS FOR IDENTIFICATION OF FLEAS

A number of species are present in birds and mammals. They are important as vectors of disease. Only one species (*Tunga persitans*) infests the skin of man.

**Rat Flea.**—This is important as vector of bubonic plague, from rat to rat and from rat to man. The fleas act as an intermediate host and vector for the dog tapeworm, *Dipylidium caninum*.

**Tunga Persitans.**—The female flea burrows into the skin of animals and birds and occasionally man. The infestation is confined to the tropics.

### METHODS FOR IDENTIFICATION OF LICE

This group of biting and sucking parasites is probably important as vectors of relapsing fever, trench fever and typhus fever. Three important varieties are found in man. The ova when laid are attached to hairs or clothing, and are spoken of as "nits." Examination of the nit under the microscope will reveal an oval structure with an operculated end, the blunt end being attached to the hair. The eggs hatch in from 7 to 10 days.

**Pediculosis Capitis, the Head Louse.**—This is found in the hair of the head. The size is from 1 to 2 mm. in length, and nits 0.6 mm. The diagnosis is made by finding the parasites or examining the hair for the nits. The infestation is spread through combs, brushes, hats, etc. For morphology see Figure 281.

**Pediculosis Corporis, the Body Louse.**—It measures from 2 to 4 mm. in length, the nits 0.8 mm. It attaches itself to the underclothing, particularly the seams. The infestation is spread through interchange of wearing apparel. This parasite is of importance as a vector of disease. For morphology see Figure 282.



**Pediculosis Pubis, the "Crab" Louse.**—It receives its name because of the claw-like extremities. It measures 0.8 to 1.2 mm. in length. It selects the hairy portion of the body about the genitalia. The ova are attached at the base of the hairs and measure 0.8 mm. The louse is usually transmitted by direct contact. Its morphology is shown in Figure 283.

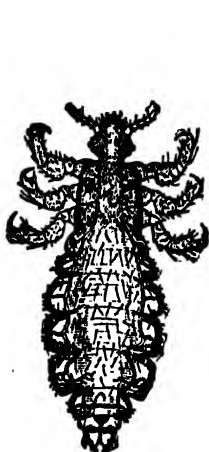


FIG. 281.—*PEDICULUS CAPITIS*

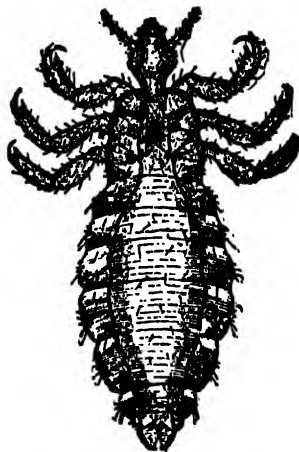


FIG. 282.—*PEDICULUS CORPORIS*



FIG. 283.—*PEDICULUS PUBIS*

(From Schamberg, *Diseases of the Skin and Eruptive Fevers*, W. B. Saunders Co., Philadelphia.)

### METHODS FOR IDENTIFICATION OF FLIES

Many species of flies are of medical interest. A large number are important as vectors of disease; others are important because of their bite.

In the skin the infestations are limited to the larval forms. At least 2 varieties actually burrow into the skin, producing tissue-destruction resembling a boil. Other species deposit eggs in open wounds or in the various cavities of the body.

The infestation of fly larvae into the skin is known as cutaneous myiasis. The chief species responsible for wound myiasis are:

The green bottle fly, *Lucilia caesar*.

The common blow fly, *Calliphora vomitoria*.

The meat or flesh flies, *Sarcophaga carnaria* and group.

The American screw worm, *Cochliomyia macellaria* (*Chrysomya macellaria*) and rarely others.

**True Cutaneous Myiasis.**—*Dermatobia hominis*, a fly indigenous to tropical America. The adult is a little over 1 cm. in length. It has a bluish-green abdomen and dull brownish wings.

The method of transmission to man is not well understood. The larvae bore their way through the skin, and in the subcutaneous tissues produce a lesion resembling a boil.

The diagnosis is made by finding the larvae upon incising the boil. When full grown the larva is 12 mm. in length. The head end is armed with a number of spines. It tapers at the posterior end.

*Cordylobia anthropaga*, the tumbu fly, indigenous to Africa. The adult is a yellowish color with black markings. The living larvae are deposited directly on the skin of animals or man. This they penetrate producing a boil-like lesion. When full grown, the larvae are about 1 cm. in length, yellowish-white in color, with an anterior pointed end provided with spines and a rather blunt posterior portion.

The diagnosis is made by demonstration of the larvae upon incising the lesion.

## **SEROLOGICAL METHODS**

### **METHODS FOR THE PREPARATION AND PRESERVATION OF PLASMA AND SERUM**

Methods for the collection of small amounts of blood by venipuncture for various serological and other laboratory examinations have been described on pages 44 to 48. In the collection of large amounts of human blood for the preparation of normal plasma, normal serum or convalescent serum for therapeutic purposes, special methods must be employed with rigid precautions against contamination with bacteria and pyrogenic substances.

#### **METHOD FOR THE COLLECTION OF HUMAN BLOOD FOR THE PREPARATION OF PLASMA AND SERUM**

The method employed by Dr. Frank W. Konzelmann at Temple University Hospital is as follows:

1. The donor should be in good health and physical condition and weigh at least 100 pounds. The systolic blood pressure should be above 100 with a hemoglobin estimation of at least 12 gms. per 100 cc. of blood. Donors with fever, abscesses, acute upper respiratory tract infections and recently extracted teeth should not be employed. Likewise those with clinical or serological evidences of syphilis. Donors with malaria should be excluded. Do not collect more than 3 cc. of blood per pound of body weight. No meals should be taken within 4 hours of collection in order to avoid chylous plasma or serum.

2. The assembled apparatus is shown in Figure 284. A Strumia blood plasma bottle of 650 cc. capacity (Kimble resistance glass) fitted with a 2-holed rubber stopper, pure gum rubber tubing (5 mm. bore) and glass tubing (7 mm. outside diameter) are employed.

3. New rubber tubing and stoppers are placed in a 5 per cent solution of sodium carbonate and autoclaved at 15 pounds pressure for 30 minutes followed by rinsing with running hot water and boiling in freshly distilled water for 5 minutes. Used rubber parts are rinsed with running tap water and the tubing cleaned by drawing through gauze at the end of 2 feet of heavy copper wire, followed by rinsing with tap and freshly distilled water. Freshly distilled water refers to that used within 3 hours after distillation.

4. Before assembly, the parts are rinsed with freshly distilled water. For the preparation of plasma 50 cc. of 4 per cent solution of sodium citrate in 0.85 per cent sodium chloride solution are placed in the bottle but omitted for the preparation of serum. The assembled apparatus is sterilized by autoclaving at 15 pounds pressure for 25 minutes. If citrate solution is used it is important to prevent excessive evaporation following sterilization by avoiding too rapid reduction of pressure in the autoclave.

5. Place the donor in a comfortable recumbent position. Select a suitable vein. Prepare the skin by cleansing with 70 per cent alcohol; apply tincture of merthiolate and cover with sterile gauze. Before puncturing infiltrate the skin over the chosen site with a sterile 1 per cent solution of procaine by means of a small sterile syringe fitted with a sterile needle of gauge 23 or 26.

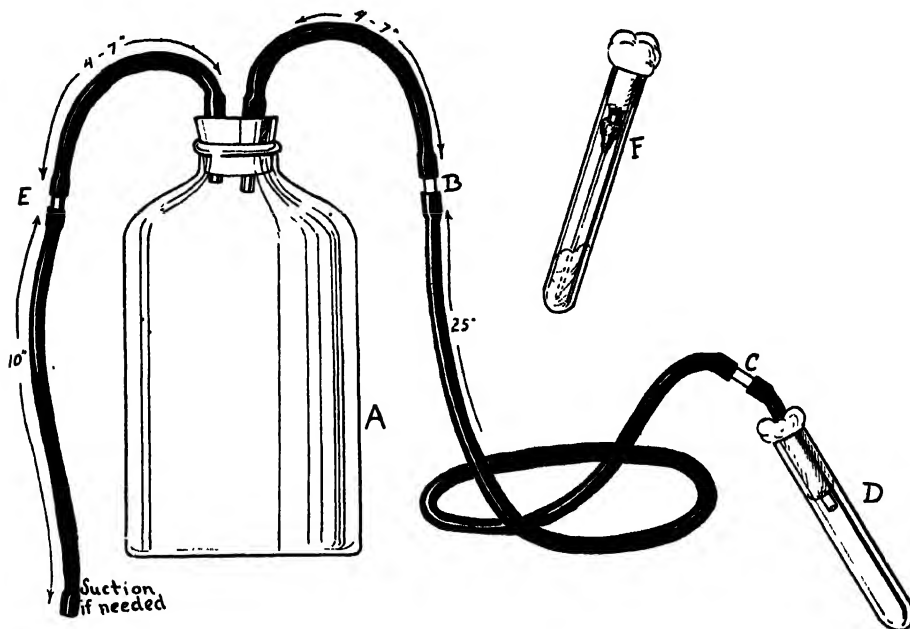


FIG. 284.—APPARATUS FOR COLLECTION OF BLOOD

A, 650 cc. bottle carrying 50 cc. citrate solution; B and C, glass observation tubes; D, metal needle adapter in a test tube stoppered with cotton; E, cotton air filter in glass tubing; F, test tube stoppered with cotton and carrying a 15-gauge needle (cotton in bottom of tube to protect point of needle.)

6. Inspect the apparatus for defects in rubber tubing and assembly. Adjust needle to the adapter. Clamp off the rubber tubing connecting the air filter to the bottle with a hemostat. Invert the bottle until the citrate solution fills the rubber tubing connected with the needle while being very careful not to allow any of the solution to flow into the tubing connected with the air filter. Clamp the tubing near the rubber stopper with a hemostat to prevent the citrate flowing back into the bottle until the needle has been introduced into the vein.

7. Apply a tourniquet and have the donor open and close the hand several times which aids in distending the vein. Introduce the needle into the vein and when blood is seen in the observation tube release the hemostat to permit the blood to flow into the bottle which is placed on the floor or below the level of the arm. Have the patient open and close the hand, which aids the flow of blood. With a 15-gauge needle suction is not ordinarily required. If citrate solution is used for the preparation of plasma, shake the bottle during collection of blood to insure thorough mixing for the prevention of coagulation and collect blood up to the 550 cc. mark. It is convenient to place the

bottle in a mechanical shaker which is stopped from time to time to determine whether or not the blood is flowing freely. If the donor appears and feels faint, give 1 or 2 teaspoonfuls of aromatic spirits of ammonia in water.

8. After obtaining the desired amount of blood, release the tourniquet with the bottle below the level of the arm, pinch the rubber tubing and remove the needle. Maintain pressure over the site of puncture with a pad of sterile gauze for at least 5 minutes and apply bandage. Allow the donor to rest and give coffee or orange juice if necessary.

9. By gentle milking discharge the blood in the rubber tubing into test tubes. Remove both rubber tubings with observation tube and air filter (B and E in Fig. 284), fold down the 2 pieces of tubing attached to the stopper and tie to the bottle or clamp each with a hemostat. For the preparation of plasma, place the blood in a refrigerator at 2 to 5° C. within an hour after collection and keep in refrigerator for 12 to 24 hours.

### METHODS FOR THE PRESERVATION OF SERUM AND PLASMA

1. Sterile serum or plasma may be kept in vials or other containers in a refrigerator without a preservative, but this is inadvisable when they are to be used for therapeutic administration. Under these conditions a preservative like 1 cc. of 1:1250 solution of phenylmercuric nitrate (basic medical grade) or merthiolate should be added to each 40 cc. (1:50,000) as a safeguard against contamination; the former is preferred because it is less likely to produce a precipitate.

2. For the preservation of positive and negative sera as controls in the conduct of agglutination, complement fixation, flocculation tests, etc., merthiolate powder is recommended in amount of 0.001 gm. per cc. or 0.1 gm. dissolved in 100 cc. It is advisable to filter the serum before the addition of merthiolate and for this purpose the Boerner centrifugal filters or the new model Seitz laboratory filters are recommended. Both employ the Seitz germicide (E.K.) filter discs and these should be washed by allowing 2 volumes of distilled water to pass through with suction continued until the disc is as dry as possible before the serum is filtered. Otherwise, the sera may be preserved by adding 0.1 cc. of 5 per cent solutions of phenol or tricresol (preferred) to each cc. of serum.

3. Guinea-pig complement serum, normal and convalescent human serum or plasma, etc., are particularly well preserved in dried powder form. For this purpose the product should be desiccated from the frozen state. This prevents denaturation of proteins and the resulting product has excellent qualities of reconstitution with sterile water and no loss of potency, generally detectable with most biological products. The process may be applied to preservation of stock cultures and of viruses as well as the immune sera and other products. Several methods have been developed during the past 10 years for carrying out the process on a practicable scale. These make use of low temperature condensation in vacuum, chemical removal of water in vacuum (chemical dessicants removing water either by chemical combination or physical adsorption), or by direct pumping. That to be used, depends upon the amount of material to be dried.

**Lyophile Method.**—In this method, devised by Florsdorf and Mudd (*Jour. Immunol.* 29: 389, 1935), the apparatus made by the F. J. Stokes Machine Company,

Philadelphia, Pa., is employed. The serum or other biologic material to be preserved is distributed with sterile precautions into glass containers. These are immersed in a bath of dry-ice in a commercial solvent (methyl cellosolve) at a temperature of approximately  $-75^{\circ}\text{C}$ . The containers of the frozen serum are attached to a manifold which leads through a condenser to a vacuum pump. The condenser is kept at  $-75^{\circ}\text{C}$ . with a bath of dry-ice in the same solvent. The whole system is rapidly evacuated and held at a pressure below 0.05 mm. of mercury by the vacuum pump. Water vapor evaporates from the frozen serum to be trapped as ice in the condenser; the rate of evaporation is sufficient to keep the serum frozen throughout the process of desiccation. The containers are sealed without breaking the original vacuum. Storage for prolonged periods should be at refrigerator temperature. The light porous residue of serum solids quickly and completely dissolves in distilled water to regenerate a serum the potency of which is not detectably different from the original serum. This procedure has been termed the "lyophile" process.

**Cryochem Method.**—In 1938, Flosdorf and Mudd described a process for accomplishing the drying of labile biological substances from the frozen state which is considerably cheaper and simpler than the lyophile process. Dry-ice is not required either for the initial freezing or for condensation of the evaporated water. A chemical, known as Drierite (anhydrous calcium sulfate, specially prepared at about  $200^{\circ}\text{C}$ .) is used for absorption of the water vapor and is repeatedly regenerable.

The containers of material are attached to the apparatus and the serum pumped essentially free of gases under low vacuum for about a half hour. A high vacuum is then established and the initial freezing is spontaneous, taking place immediately as a result of the particularly rapid dehydrating action of the Drierite. During the desiccation, not as high a vacuum is required as in the lyophile process and completion of the drying is accomplished in a shorter time.

In many cases the final product has superior solubility and other characteristics, but presents a somewhat less attractive appearance. In cases where appearance might be a major factor, the serum may be frozen initially with dry-ice or other means.

The apparatus (Fig. 285) and Drierite may be purchased from the F. J. Stokes Machine Co., Philadelphia, Pa. In regeneration of the Drierite, with smaller units, the ordinary hot air sterilizing ovens may be used for driving off the moisture. The larger models of the apparatus are equipped with built-in electrical regeneration units so that the chemical need not be removed from the apparatus. When the Drierite has been used to capacity, the electric switch is turned on for regeneration. After a few hours, the material is allowed to cool overnight and is then ready for further use.

At no time is dry-ice storage required, much less attention is needed, and there is no increased cost resulting from sub-capacity operation. These and other factors, as well as the low general cost, make this process much more practical for clinical laboratory use. In many foreign countries, where dry-ice is unavailable, this type of equipment has provided the only solution to the problem of simple and economical use of desiccation from the frozen state.

**Desivac Method.**—In 1940, Flosdorf, Stokes and Mudd described the use of direct pumping as a simple economical procedure for carrying out desiccation from the frozen state on a larger scale than generally is required for such products as guinea-pig complement, immune sera, and bacterial cultures. It is admirably suited, however, for

production of normal plasma and normal serum for use as a blood substitute in transfusion because in this case, the volume of material which must be processed is so much larger. The machine (Fig. 286) may be purchased from the F. J. Stokes Machine Co., Philadelphia, Pa. The direct pumping is carried out either by means of mechanical pumps equipped with a special centrifugal clarifier for elimination of the condensed water from the oil in the pump or by means of steam ejectors. The former have limitations with regard to capacity but for use in many hospitals, where production is

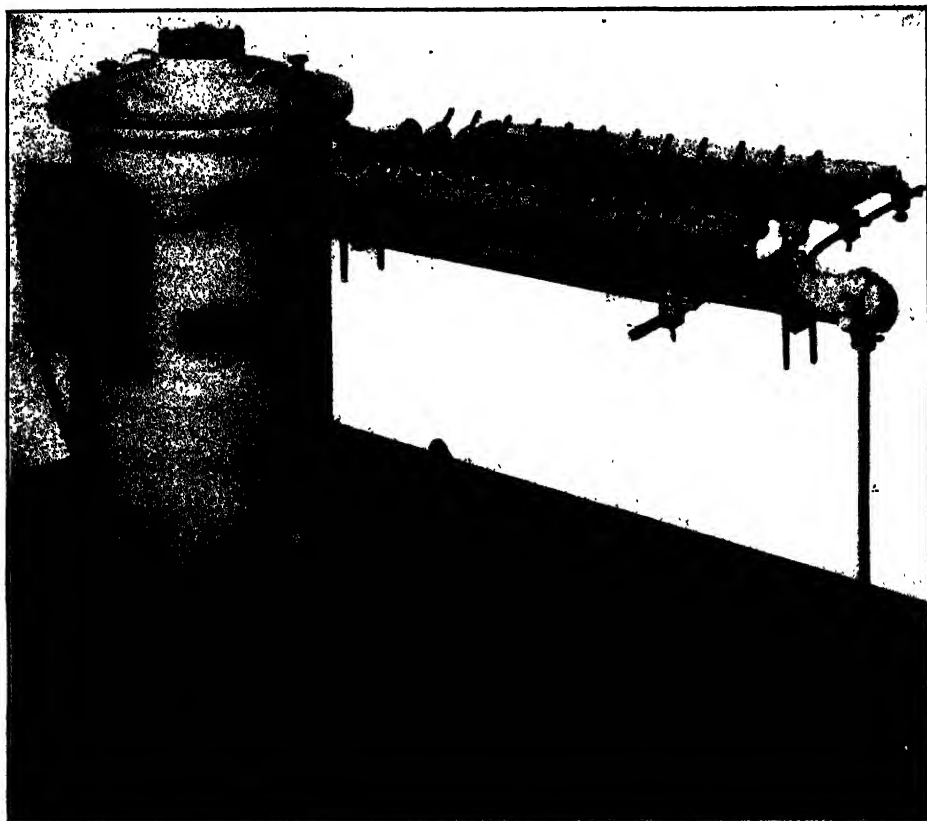


FIG. 285.—CRYOCHEM MACHINE

Capacity one liter per day. Suitable for general laboratory purposes in the preservation of plasma, complement, immune sera, cultures, etc. (Courtesy of Dr. E. W. Flosdorf.)

limited, their capacity is ample. In commercial production, however, the use of steam ejectors in direct pumping has been proved to be economical. In the case of production of plasma by individual hospitals where a capacity greater than that provided by mechanical pumps is required, low temperature mechanically refrigerated condensers may be used advantageously in place of the dry-ice condensers as originally described by Flosdorf and Mudd in the lyophile process. Otherwise, the method of processing is the same. Such mechanically refrigerated condensers are also being used commercially for the large scale production of plasma.

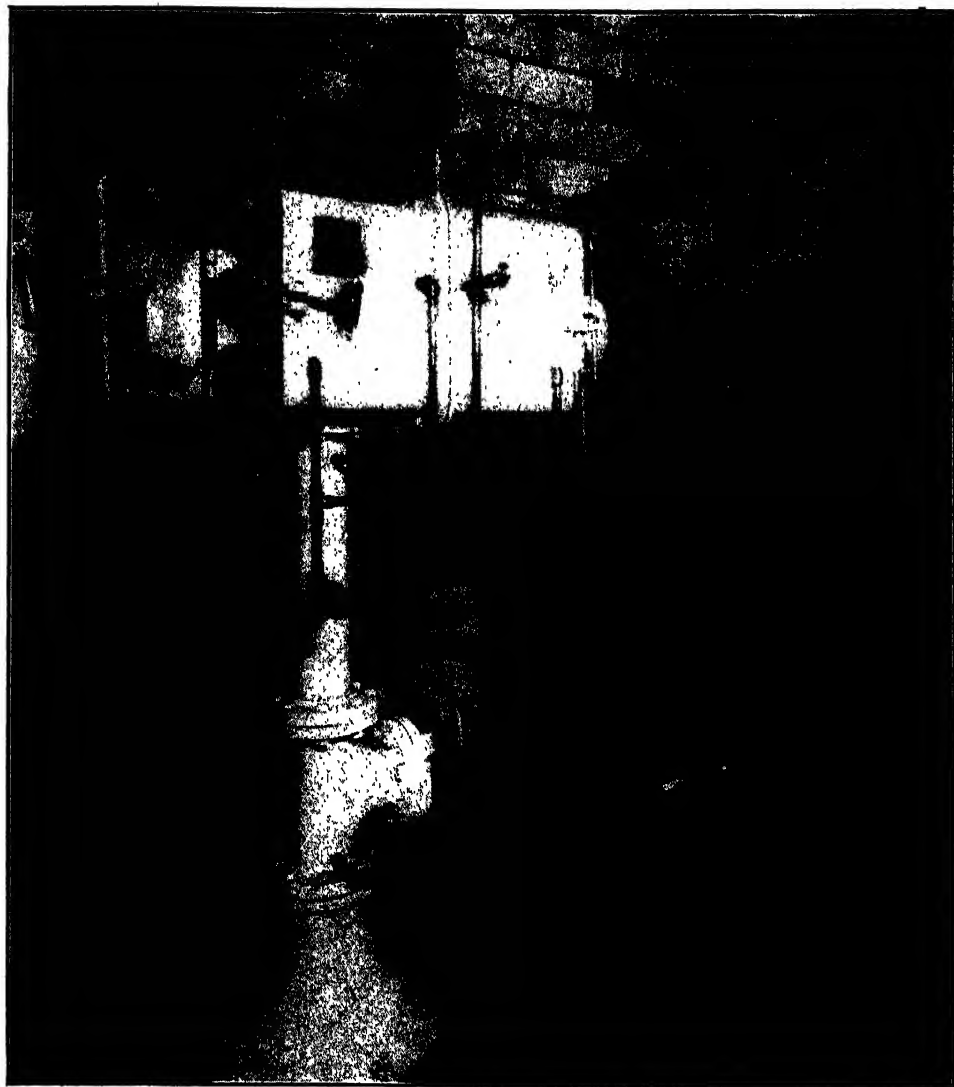


FIG. 286.—DESIVAC MACHINE

For the production of about 40 bottles of plasma (300 cc. each) per week. Dry-ice or chemicals are not required. May be equipped for pre-freezing mechanically within the drying chamber so that a shelling machine is not required. In this case, freezing and drying are carried out as a single operation. (Courtesy of Dr. E. W. Flösdorf.)

### **METHOD FOR THE PREPARATION OF NORMAL HUMAN PLASMA**

The following method is according to that of Strumia as modified by Dr. Frank W. Konzelmann for the preparation of normal plasma in Temple University Hospital.

1. The blood is collected as previously described (page 616).
2. The bottles of citrated blood are very carefully balanced in cups and centrifuged at 2500 r.p.m for 45 minutes. Upon completion, the rheostat is gradually turned down; the centrifuge brake is not applied.



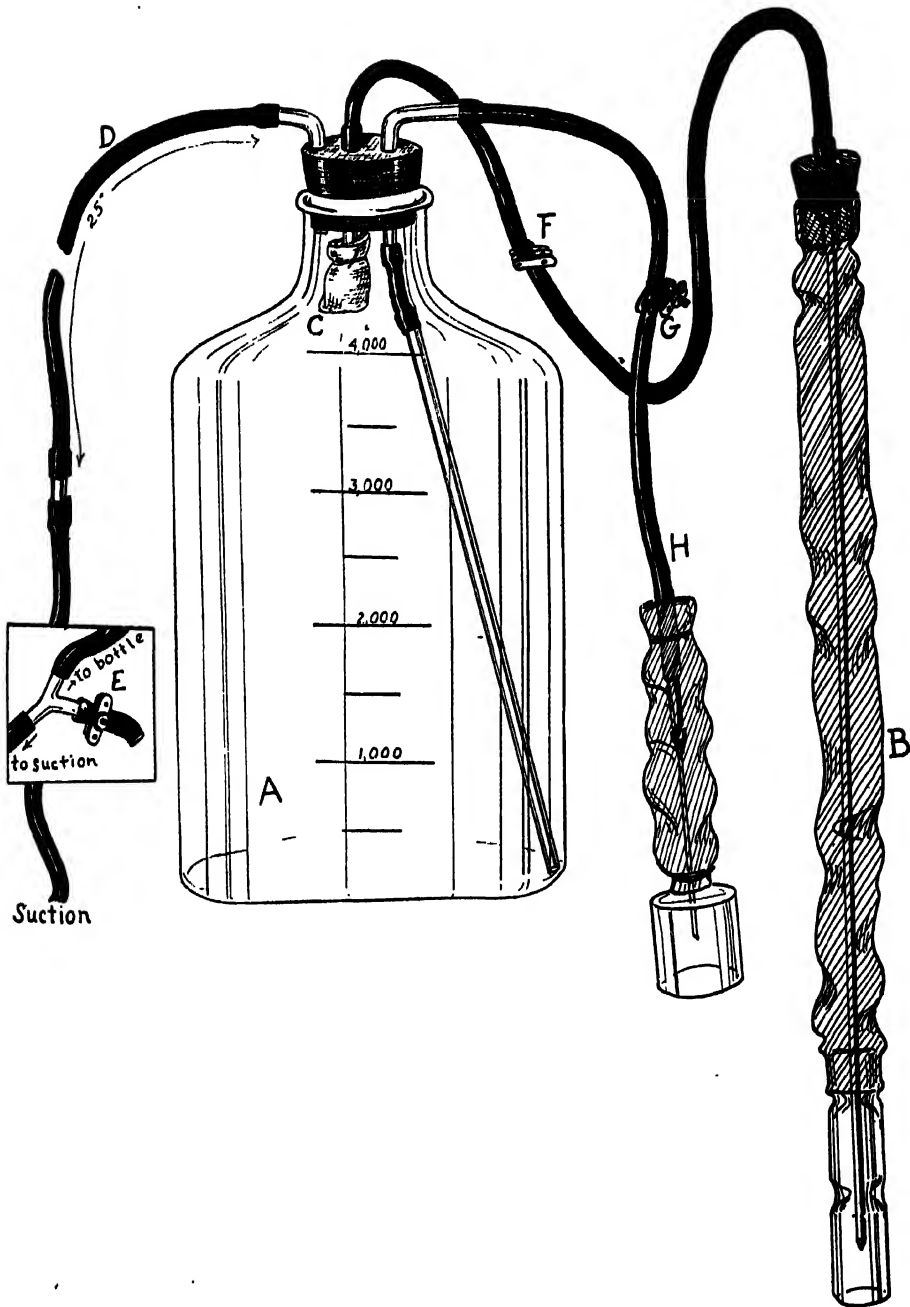


FIG. 287.—APPARATUS FOR POOLING AND DISTRIBUTING PLASMA

A, pooling bottle; B, aspirating cannula; C, sterile gauze filter; D, tubing with cotton air filter for exhausting air from pool bottle; E, F and G, one-inch Hoffmann clamps; H, distributing apparatus.

3. The plasma from each bottle (totalling not more than 13 bottles) is now aspirated into a 4-liter bottle for pooling purposes. The parts of the apparatus are first washed in freshly distilled water, assembled (Fig. 287), wrapped in muslin and autoclaved at 15 pounds pressure for 25 minutes. The operator wears a sterile cap, gown, face mask and rubber gloves, working in a closed room. A rigid aseptic technic is required. The aspirating cannula for the removal of plasma from the bottles of blood is encased in gooch crucible rubber tubing (1 inch outside diameter) to which is attached a glass bell with two constrictions (length 9.5 cm.; outside diameter 1.7 cm.). The aspirated plasma is filtered through 4 layers of 40-mesh gauze (previously boiled in freshly distilled water) as it flows into the pooling bottle. Aspiration is conducted under good light to avoid mixture of corpuscles with the plasma. Air is exhausted from the pooling bottle by means of suction. During aspiration clamp F is opened and clamp G is closed (Fig. 287). The heads of the centrifuged bottles are sterilized by placing on them sterile gauze sponges saturated with 5 per cent solution of tricresol and the rubber tubing covered with sterile alcohol sponges for at least 5 minutes. Upon completion of the aspirations the plasma is well mixed by rotation.

4. The pooled plasma is now distributed in amounts of 300 cc. in sterile Strumia blood plasma bottles (400 cc. capacity) made of Kimble resistance glass. For this purpose the pooling bottle is fitted with a distributing apparatus (H in Fig. 287) composed of rubber tubing, an adapter, spinal puncture needle (gauge 16 to 18) and a glass bell. The plasma bottles are fitted with perforated rubber stoppers covered with rubber hoods. Each is prepared by rinsing with freshly distilled water (leaving 1 cc.), exhausting the air by pump suction for 5 minutes and autoclaving at 15 pounds pressure for 25 minutes. The vacuum within each bottle serves for the aspiration of the pooled plasma. Each bottle is now labelled and 7.5 cc. of a 1:1250 solution of phenylmercuric nitrate added as a preservative which gives a final dilution of approximately 1:50,000 in 300 cc. of plasma.

5. Inoculate a flask of 50 cc. of Brewer's sodium thioglycollate broth with 5 cc. of the pooled plasma for anaerobic cultivation; also a flask of 50 cc. of veal infusion broth with 5 cc. for aerobic cultivation and place 20 cc. in a dry sterile bottle designated as the "pilot bottle." The cultures are incubated at 37° C. for 15 days. The "pilot bottle" is left at room temperature for 10 days when the plasma is cultured aerobically and anaerobically. In the meantime, the bottles of plasma are kept at —20° C. or desiccated by the desivac method, previously described. It is dispensed for administration only in case all cultures of the pooled plasma have proven sterile.

#### **METHOD FOR THE PREPARATION OF NORMAL AND CONVALESCENT HUMAN SERUM**

1. Pooled normal human sera are used by the armed forces of Canada and Great Britain instead of normal human plasma with equally good therapeutic results.

2. Sterile human convalescent sera are frequently employed for prophylactic and therapeutic purposes in measles, scarlet fever, mumps, chickenpox, etc. Blood may be obtained from donors following recovery or when convalescence is well established.

3. The method for collection of blood is essentially the same as that described on page 616, except that sodium citrate is not employed for the prevention of coagulation.

4. Allow the blood to stand at room temperature for several hours and in a refrig-

erator overnight for the separation of serum. The bottles of blood may be then "rimmed" and centrifuged and the sera pooled, distributed, cultured and preserved in the same manner as described above for the preparation of plasma. The finished serum should be crystal clear and free of erythrocytes, fibrin and fats, as well as being sterile and Wassermann-negative. It may be kept in a fluid state or desiccated by the cryochem or desivac methods, previously described.

## METHODS FOR CONDUCTING BACTERIAL AGGLUTINATION AND OPSONOCYTOPHAGIC TESTS

**Principles.**—1. The bacterial agglutination tests have proven valuable in the serum diagnosis of many diseases of man and the lower animals with special reference to typhoid and paratyphoid fevers, brucellosis, tularemia, leptospirosis, typhus fever, Rocky Mountain spotted fever and other rickettsial diseases. They are likewise helpful in the diagnosis of pertussis, bacillary dysentery, bubonic plague, glanders, etc., as well as in the detection of bacterial carriers with special reference to typhoid and paratyphoid bacilli.

2. To be of diagnostic value the titers of agglutination reactions must be definitely higher than those due to natural agglutinins normally present in sera.

3. Immune agglutinins are usually produced by the administration of vaccines in the prophylaxis and treatment of disease. Since they may persist for variable periods of time their occurrence may complicate the interpretation of agglutination tests, especially in relation to typhoid and paratyphoid fevers occurring in previously vaccinated individuals.

4. Owing to biological relationships among some of the bacteria, group agglutination reactions may occur resulting in diagnostic errors. Under these circumstances the bacterial antigen giving the highest titer usually indicates the nature of the infection; otherwise, absorption agglutination tests may be required.

5. In some instances agglutinins may be produced through stimulation by a new and unrelated infection producing the *anamnestic reaction* as, for example, the temporary production of typhoid agglutinin during an attack of influenza or some other unrelated acute infectious disease.

6. As far as possible antigens for the conduct of agglutination tests should be prepared of "smooth" strains of the micro-organism. Selected agglutinable strains should be chosen. Freshly isolated micro-organisms may not be agglutinable. Others, like streptococci and meningococci, may undergo spontaneous agglutination leading to possible errors in the reading and interpretation of reactions.

7. With some of the motile bacilli, agglutination tests are conducted best routinely with antigens prepared with formalin (flagellar or H antigen) and with alcohol (somatic or O antigen); this is particularly true in the case of the typhoid and paratyphoid bacilli.

8. *In the preparation of bacterial antigens and especially those of P. tularensis, Br. abortus and Br. melitensis, great care must be exercised against accidental laboratory infections.*

9. Prozone or agglutinoid reactions may occur in which the lowest dilutions of serum show no agglutination at all or less than produced by higher dilutions. Therefore, quantitative tests are always advisable as a safeguard against possible diagnostic errors.

10. In the laboratory, agglutinins occurring in specific immune sera are extremely valuable in agglutination tests for the final identification of pneumococci, beta hemolytic streptococci, meningococci, typhoid, paratyphoid and dysentery bacilli, etc., as previously described.

11. Agglutination tests are only of limited value in the diagnosis of actinomycosis, sporotrichosis, moniliasis and other mycotic diseases. This is due to the low

production of agglutinins and difficulties experienced in the production of suitable antigens.

12. Agglutination tests are practically of no value as aids in the diagnosis of protozoal diseases like leishmaniasis and trypanosomiasis.

13. Hemagglutination tests are of great value in relation to the selection of donors for blood and plasma transfusions, the diagnosis of infectious mononucleosis and for medico-legal purposes in relation to paternity; these are given in this section.

## METHODS FOR THE PREPARATION AND TITRATION OF ANTIGENS

**Living Antigens.**—Antigens of living micro-organisms are not commonly employed except in the conduct of the microscopic Widal or agglutination test for typhoid and paratyphoid fevers. Actively motile organisms of smooth colonies are preferred. The cultures of typhoid or paratyphoid bacilli (A and B) may be grown in broth for 18 to 24 hours. At 25° C. (on top of the incubator) longer forms usually occur. Do not shake the culture and use upper part. Stock cultures on agar slants may be kept in a refrigerator and transplanted at intervals. When tests are frequently conducted the broth cultures should be subcultured daily. Gilbert and Coleman recommend 2- to 4-hour cultures in bile peptone solution. The culture should be free of clumps or spontaneous agglutination (Figs. 288 and 289).

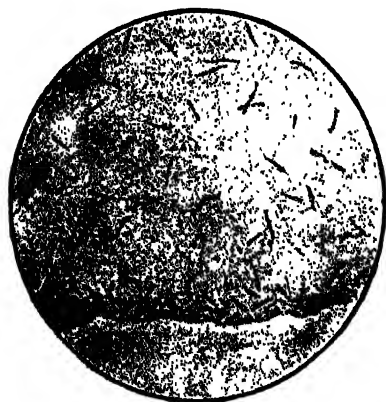


FIG. 288.—A SATISFACTORY ANTIGEN FOR MICROSCOPIC WIDAL TEST



FIG. 289.—AN UNSATISFACTORY ANTIGEN FOR MICROSCOPIC WIDAL TEST

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

**Phenolized Antigens for Macroscopic Slow Agglutination Tests.**—1. Cultivate the micro-organism on a suitable solid medium for 48 to 72 hours. Examine stained smears and exclude growths showing contamination.

2. Wash off the growths with small amounts of 0.5 per cent phenol in normal saline solution. Due care should be taken to avoid particles of culture medium.

3. Transfer the suspensions to a sterile flask carrying glass beads and shake sufficiently to secure an even suspension.

4. If necessary, filter through several layers of sterile cheesecloth or paper to remove large clumps of bacteria and particles of culture medium.

5. An alternate method consists in cultivating the micro-organism in a suitable fluid medium. Examine stained smears and exclude growths showing contamination. Carefully transfer the culture to centrifuge tubes and centrifuge sufficiently at high speed. Discard the supernatant fluid, suspend the sediment of bacteria in phenolized saline solution, shake with glass beads and filter if necessary.

6. Dilute the suspension with phenolized saline solution to correspond approximately to about 3000 million per cc. or slightly more than the density of stock typhoid-paratyphoid vaccine.

7. Incubate at 37° C. for 24 hours during which sterilization usually occurs if spores are absent.

**Heat-Killed Antigens for Macroscopic Slow Agglutination Tests.**—These are prepared in the same manner as phenolized antigens except that the suspensions are diluted to the desired concentration with sterile saline solution and heated in a water bath at 60 to 65° C. for 1 hour. To each 90 cc. add 10 cc. of a 5 per cent solution of phenol or tricresol (final concentration of 0.5 per cent) as a preservative. Alternate preservatives consist of adding 10 cc. of 1:1000 merthiolate to 90 cc. of suspension (final concentration 1:10,000) or 0.1 cc. of formalin to 100 cc. of suspension.

**Formalized Antigens for Macroscopic Slow Agglutination Tests.**—This method is generally preferred for the routine preparation of antigens. In the case of motile bacilli it yields the so-called H or flagellar antigen.

1. Cultivate the micro-organism on a sufficient number of slants of suitable medium at 37° C. for 24 to 72 hours. Prepare and examine smears of each stained by the method of Gram and discard those showing contamination.

2. With a sterile pipet, add 3 to 5 cc. of sterile saline solution to each slant and remove the growths. Transfer the suspensions to a sterile flask, carefully avoiding contamination.

3. With a sterile pipet fitted with a cotton plug to which is attached a 12 to 15 inch piece of rubber tubing with a mouthpiece, transfer 10 cc. of suspension to each of a sufficient number of Blake bottles of suitable solid medium. Spread the suspension over the surface of each bottle and incubate at 37° C. for 24 to 72 hours with the bottles tilted so that the excess fluid collects at the bottom.

4. Examine the bottles and discard any showing gross contamination. Allow the remaining ones to lie flat for 1 or 2 hours so that the surface of each will be entirely covered by the fluid. The bottles are then rocked back and forth until each growth is well emulsified. Prepare and examine a smear of each stained by the method of Gram and discard those showing contamination.

5. Remove each suspension with a sterile pipet to a sterile flask or bottle. To each 99.5 cc. add 0.5 cc. of formalin. Keep at room temperature in the dark and culture daily for sterility (several days are usually required). When sterile, dilute with sufficient 0.5 per cent solution of formalin in normal saline solution to give about 3000 million per cc. or slightly denser than stock typhoid-paratyphoid vaccine.

6. An alternate method which is particularly useful in preparing antigens of *P. tularensis*, *Br. abortus* and *Br. melitensis* consists in inoculating Blake bottles by dipping sterile swabs in the suspension, squeezing out the excess by pressure against the inside of the bottles and then quickly rubbing the swab over the surface of the medium, the slight roughening of which is helpful in obtaining heavier growths. After incubation the growths are harvested by adding 10 to 15 cc. of 0.5 per cent formalin

in saline solution to each bottle and allowing the fluid to remain on the surface for about an hour or until the growth is loosened. The emulsions are then removed, mixed, and filtered through several layers of sterile gauze to remove small particles of medium before the antigen is completed, as described above. In the case of *Brucella abortus* antigen the suspension should be heated in a water bath at 60° C. for 1 hour.

**Formalized Antigens for Rapid Slide Agglutination Tests.**—1. The technic is the same as described above except that not more than 5 cc. of 0.5 per cent formalin in 12 per cent sodium chloride solution are added to each Blake bottle.

2. Remove the suspensions, mix, filter through at least 8 layers of sterile gauze, and centrifuge at high speed in graduated centrifuge tubes for 1 or more hours to pack the micro-organisms as completely as possible.

3. Decant and save the supernatant fluid (to be used as a diluent in titrating the antigen).

4. To each 1 cc. of packed micro-organisms add 5 to 7 cc. of the supernatant fluid and mix thoroughly.

5. In the case of *Br. abortus* and *Br. melitensis* antigens, remove the growths from the Blake bottles with 0.5 per cent formalin in a solution containing 12 per cent sodium chloride and 20 per cent glycerin. Heat in a water bath at 60° C. for an hour before filtering, centrifuging and completing the antigen. Huddleson advises placing the suspensions in a pyrex beaker, boiling slowly for 10 minutes and cooling rapidly before filtering, centrifuging and completing the antigen. The reaction should be adjusted to pH 6.8.

**Alcoholic Antigens for Macroscopic Slow Agglutination Tests.**—Antigens prepared with alcohol are of the somatic or O type.

1. The technic is the same as described for the preparation of formalized or H antigens except that Blake bottles are inoculated with swabs, as previously described, and the growths removed after incubation by adding 10 cc. of 0.5 per cent phenol in normal saline solution to each bottle instead of formalized saline solution.

2. The mixed suspensions in a flask are then treated with sufficient absolute or 95 per cent ethyl alcohol, while constantly rotating the flask, to give a final concentration of about 54 per cent alcohol.

3. After incubation overnight at 37° C., decant the supernatant fluid into a sterile bottle or flask and discard the sediment.

4. Culture for sterility and calculate the dilution required to give a density slightly greater than that of stock typhoid-paratyphoid vaccine. As a diluent, use a solution of alcohol in normal saline solution sufficient to give a 2.5 per cent final concentration of alcohol in the finished antigen.

**Alcoholic Antigens for Rapid Slide Agglutination Tests.**—1. The technic is the same as described for the preparation of formalized antigens for the rapid slide test except that not more than 5 cc. of a 12 per cent solution of sodium chloride are added to each Blake bottle.

2. After centrifugation the supernatant fluid is discarded and the packed micro-organisms pooled, using sufficient normal saline solution for transfer purposes.

3. Place the concentrated suspension in a flask and add 15 to 20 volumes of 95 per cent alcohol with vigorous shaking for 10 minutes.

4. Incubate at 37° C. for 24 hours.

5. Siphon off and discard the alcohol from the white flocculent mass. Centrifuge in graduated tubes and remove the balance of the alcohol.

6. To each 1 cc. of packed micro-organisms add about 5 to 7 cc. of 12 per cent sodium chloride solution.

7. Add sufficient 1 per cent aqueous solutions of gentian violet and brilliant green to give a final concentration of 1:40,000 of the former and 1:20,000 of the latter. These dyes prevent contamination of the antigens during use and facilitate the reading of reactions.

**Titration of Antigens.**—It is always advisable to titrate antigens for agglutinability with known positive human or rabbit immune sera. Also with known normal human sera for spontaneous agglutination or other causes for nonspecific positive reactions.

*Antigens for Slow Macroscopic Tests.*—The technic of titration of phenolized, heat-killed and formalized antigens is the same as that given below for the Routine Slow Macroscopic Agglutination Test except that 2 rows of tubes are used, the first carrying a series of dilutions of known positive serum and the second a series of similar dilutions of known negative serum.

*Antigens for Rapid Slide Agglutination Tests.*—Formalized and alcoholic antigens for these tests may be titrated as follows:

1. In a series of 4 small test tubes prepare 1:2, 1:4, 1:8 and 1:16 dilutions of antigen. In the case of a formalized antigen use the supernatant fluid retained in its preparation as the diluent. For alcoholic antigens use a 2.5 per cent solution of absolute ethyl alcohol in saline solution as the diluent.

2. In a series of 4 small test tubes prepare 1:40, 1:80, 1:160 and 1:320 dilutions of a known positive serum, using normal saline solution as the diluent. At the same time prepare 4 similar dilutions of a known negative serum.

3. With a wax pencil, divide each of 4 slides into 2 rows of 4 squares each.

4. Place a 5 mm. loopful of antigen 1:2 in each of the 8 squares of a slide. To each of the upper 4 squares add a loopful of 1:40, 1:80, 1:160 and 1:320 dilutions of positive serum respectively. To each of the 4 lower squares add a loopful of the 4 dilutions of negative serum respectively.

5. Gently rock the slide back and forth 15 to 20 times to mix the antigen and sera. The final dilutions of each serum are now 1:80, 1:160, 1:320 and 1:640 respectively.

6. Read the reactions at once while holding the slide over a desk or other lamp, so that the light is transmitted through the slide but not directly into the observer's eyes.

7. Positive reactions may be recorded as 4 + (complete agglutination), 3 + (75 per cent agglutination), 2 + (50 per cent agglutination), and 1 + (25 per cent agglutination). Negative reactions should be observed with the normal serum unless it contains sufficient natural agglutination to give a positive reaction with the 1:80 dilution.

8. Set up similar slides with the 1:4, 1:8 and 1:16 dilutions of antigen.

9. The highest dilution of antigen showing satisfactory agglutination with final dilutions of positive serum 1:160 or higher is the dilution to employ. The antigen should be diluted to this concentration with the diluent employed in the titration.



**ROUTINE SLOW MACROSCOPIC AGGLUTINATION TEST**

1. The serum should be clear, free of erythrocytes and not too heavily colored with hemoglobin. If necessary, centrifuge the specimen.
2. Arrange 10 small test tubes in a rack.
3. Place 0.9 cc. of normal saline solution in the first tube and 0.5 cc. in each of the remaining tubes.
4. Place 0.1 cc. of serum in the first tube; mix, transfer 0.5 cc. to tube No. 2; mix, transfer 0.5 cc. to tube No. 3 and so on to tube No. 9 from which 0.5 cc. is discarded.
5. To each tube add 0.5 cc. of antigen (well shaken) and mix thoroughly. The final dilutions of serum in the first nine tubes are now 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and 1:5120 respectively. Tube No. 10 is the antigen control.
6. It is advisable to set up tests employing known positive and negative sera at the same time and in the same manner.
7. Incubate at 55° C. (preferably in a water bath) overnight (15 to 18 hours) followed by 2 hours in a refrigerator before reading and recording the reactions.



FIG. 290.—POSITIVE MACROSCOPIC AGGLUTINATION REACTION

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

8. The antigen control should show no agglutination. It should be uniformly turbid. If there is any settling at all, it should show only as a small deposit in the bottom of the tube which is readily resuspended by gentle mixing. Examine the negative serum control to eliminate any possible nonspecific or spontaneous agglutination in dilutions other than those considered within the normal limits of agglutination due to natural agglutinin. The positive controls should show marked agglutination indicative of a satisfactory sensitivity of the antigen.

9. Positive reactions (Fig. 290) may be read and recorded for each tube as follows.

Complete agglutination with perfectly clear supernatant fluid	= 4 +
Marked agglutination with slightly turbid supernatant fluid	= 3 +
Moderate agglutination with turbid supernatant fluid	= 2 +
Slight agglutination with turbid supernatant fluid	= 1 +

With phenolized, heat-killed and formalized antigens the agglutinated micro-organisms occur as flocculent sediments; with alcoholic antigens the sediments are granular and less voluminous. To examine sediment, hold the tube rigid at the top and gently tap the bottom just sufficient to stir up the sediment which appears as masses or clumps in positive reactions. Too much agitation may re-suspend the antigen.

10. The highest final dilution of serum showing a + 2 reaction may be regarded as the titer.

### ROUTINE RAPID SLIDE AGGLUTINATION TEST

1. In a series of 7 small test tubes prepare 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 dilutions of serum with normal saline solution.

2. With a wax pencil divide a slide into 2 rows of 4 squares each.

3. Place a 5 mm. loopful of each of the 7 dilutions of serum on the first 7 squares respectively; on the eighth square place a loopful of saline solution (antigen control).

4. Add a loopful of antigen to each square. Gently rock the slide back and forth 15 to 20 times. The final dilutions of the serum in the first 7 squares are now 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 respectively.

5. Read the reactions at once while holding the slide over a desk or other lamp, so that the light is transmitted through the slide but not directly into the observer's eyes.

6. The eighth square (antigen control) should show no agglutination. Positive reactions may be recorded as 4 + (complete agglutination), 3 + (75 per cent agglutination), 2 + (50 per cent agglutination) and 1 + (25 per cent agglutination). The highest final dilution of serum giving a 2 + reaction may be regarded as the titer of the serum being tested.

7. It is advisable to conduct duplicate tests with known positive and negative sera as controls.

8. When large numbers of sera are to be tested with typhoid and paratyphoid antigens a *screen test* is permissible. This is conducted by using a loopful of antigen and a loopful of undiluted serum. Negative reactions may be reported, but all sera showing positive reactions must be re-tested by the above quantitative method, or preferably by the slow macroscopic method described on page 630.

**Huddleson Rapid Slide Agglutination Test.**—This test affords a rapid and satisfactory method for conducting agglutination tests for brucellosis in human beings or cattle, using an antigen of *Br. abortus*. The following are required: (a) Serological pipets of 0.2 cc. capacity graduated to 0.01 cc.; (b) a plate of double thickness window glass (14 by 6 inches) ruled with a diamond point into inch squares (12 horizontally and 5 vertically); and (c) a darkfield illumination box (Fig. 291), although it may be dispensed with if the glass plate is placed upon an ordinary laboratory table with a black background. The technic is as follows:

1. Separate the sera from the clots and centrifuge if necessary to remove corpuscles. Use unheated.

2. *The darkfield box should be placed where it is not too intensely lighted, as too much light from above interferes with the indirect lighting of the plate from inside the box.* A desk lamp is of great advantage while the serum and antigen are being placed

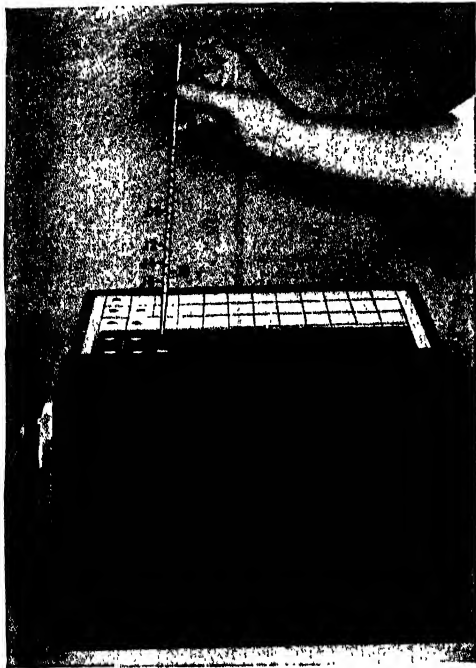


FIG. 291.—DARKFIELD ILLUMINATION BOX; PLACING SERUM (HUDDLESON)

on the plate but it should be turned off when the tests are ready to be read so as not to interfere with the indirect lighting from the box. *Do not turn on the light in the box until ready to read the test as the plate will otherwise become too warm.*

3. Arrange the serum samples in a row parallel with the box. The glass plate, with the etched squares upward, is placed over the opening of the box and the identification number of the serum sample marked with a wax pencil on either the top or bottom of the row of squares used.

4. With a clean 0.2 cc. pipet, draw up serum from the first blood sample to the zero mark on the pipet. Beginning in the bottom left-hand square of the plate, place the following amounts of serum in the succeeding squares towards the top (see Fig. 291):

*Reading on Pipet*

1st square (0.08 cc.)	0.08
2nd square (0.04 cc.)	0.12
3rd square (0.02 cc.)	0.14
4th square (0.01 cc.)	0.15
5th square (0.004 cc.)	about midway, 0.15 and 0.16

5. This manner of placing the serum brings the smallest amount farthest from the heat of the electric bulb, reducing the rapidity of drying of the smallest amounts of serum. The procedure is continued, using the next set of vertical squares and a separate pipet for each sample. The best results are obtained by testing only 4 or 5 samples at a time, as otherwise the small amounts of serum dry out too much before the test is completed.

6. If the pipet has been placed deep in the serum, there will be some serum which will collect on the outside at the tip. For accuracy, this should be removed by touching the tip of the pipet against the lip of the vial.

7. After thoroughly shaking the vial, remove a dropper full of the antigen. Holding the dropper in a vertical position, add 1 drop to each amount of serum on the

plate. *Care should be taken to hold the dropper in a vertical position since holding it at another angle will make a considerable difference in the amount of antigen delivered. Always replace the dropper directly in the vial of antigen (Fig. 292).*

8. The final dilutions are now 1:25, 1:50, 1:100, 1:200 and 1:500.

9. With a clean toothpick mix the serum and antigen, using a new toothpick for each sample. Always start at the top of the plate in the square containing the smallest amount (0.004 cc.) of serum and continue downward to the largest amount. Spread the mixture over about  $\frac{3}{4}$  the area of the square without coming in contact with the etched dividing lines.

10. Immediately after the samples have been mixed, remove the plate from the box and tilt slightly backward and forward slowly for about 2 minutes. Place the plate on the box, turn on the light and record the results. When working without the box, make the readings against a light so that the plate is illuminated from beneath.

11. The reactions stand out very clearly as shown in Figure 293. It is not difficult

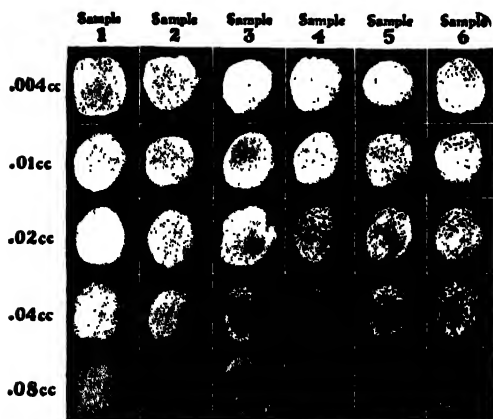


FIG. 293.—DIFFERENT DEGREES OF AGGLUTINATION REACTIONS IN BRUCELLOSIS (HUDDLESON)

12. Immediately after using, the pipets should be rinsed several times with fresh water until thoroughly clean. Then boil in distilled water and drain all the water out before using again. The glass plate may be cleansed with cleaning powder and brush, after which it is rinsed with distilled water and dried. By having several clean plates available, one can proceed with the testing of additional samples without delay. *Absolute cleanliness of glassware is essential.*

13. With human sera a positive reaction in a titer of 1:100 is considered diag-

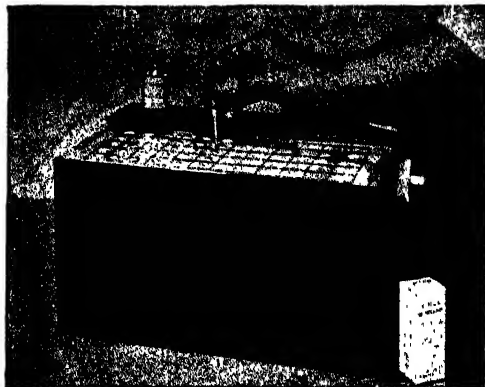


FIG. 292.—AGGLUTINATION TEST FOR BRUCELLOSIS; ADDING ANTIGEN (HUDDLESON)

to distinguish between complete clumping of the antigen and different degrees of incomplete clumping, *e.g.*, in sample 6 there is complete agglutination in all amounts of serum, while in sample 4 the clumping is complete in only the last 3 amounts. A negative serum (sample 1) causes no flocculation of the antigen. There are often encountered, however, sera which produce a trace of flocculation in the 0.08 cc. amount. In the "slow" or test tube method, this occurrence will often pass unobserved, unless viewed with a hand lens. This type of clumping appears to be due to the presence of native agglutinins in the serum.

nostic of undulant fever. With cattle sera a positive reaction in a dilution of 1:50 is suspicious of present or past infection, while positive reactions in 1:100 or higher are diagnostic. Agglutinins generally appear in the blood after the infection has been present for a week or longer.

14. This test can be conducted with *milk* from cows for the detection of those carrying *Brucella abortus* in the udders as follows:

(a) Collect separate samples of the fore milk from each quarter, using clean, sterilized vials of about  $\frac{1}{2}$  ounce capacity. Before collecting the samples, place in each vial a small amount of rennin powder (about what can be picked up between the prongs of a small pair of forceps or on the tip of a small knife blade). Then draw the milk directly into the vial and place in a slanting position. The whey will separate out in about an hour at room temperature.

(b) The test is conducted with the whey in exactly the same manner as previously described for blood sera.

### MICROSCOPIC AGGLUTINATION TEST EMPLOYING LIVING CULTURES

This test may be employed in conducting the Widal or agglutination test for typhoid and paratyphoid fevers, but has been largely replaced by the macroscopic slow agglutination test employing formalized (flagellar or H) and alcoholic (somatic or O) antigens. Since living cultures are employed, due care must be exercised and the slides, cover-glasses, etc., placed in a disinfectant solution or boiled for 5 minutes before handling and cleaning. The working table should be wiped with a disinfectant solution. The technic may be as follows:

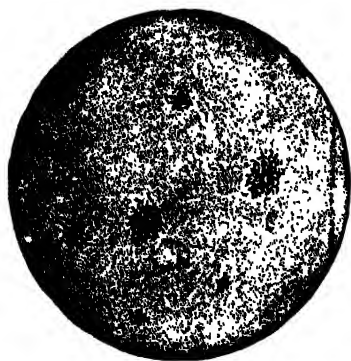


FIG. 294.—A POSITIVE WIDAL REACTION (WOOD)

1. Take 2 small watch crystals, hollow slides, or small test tubes and place 0.05 cc. of serum in each.

2. Add to one 1.0 cc. of normal salt solution and to the other 2.0 cc., making dilutions approximately 1:20 and 1:40 respectively.

3. Place 1 loopful of culture of typhoid bacillus antigen (page 626) in the middle of each of 3 cover-glasses.

4. To the first, mix 1 loopful of serum diluted 1:20. To the second, mix 1 loopful of serum diluted 1:40. To the third, mix 1 loopful of normal salt solution which is the antigen control.

5. Mount each in vaselin on hanging drop slide. The final dilutions obtained are 1:40 and 1:80; therefore, mark the slides as follows: No. 1, 1:40; No. 2, 1:80; and No. 3 control.

6. Make similar preparations with the paratyphoid cultures.

7. Allow to stand at room temperature or preferably in an incubator at 37° C. for an hour.

8. Examine with 1/6 objective using very subdued light. Controls should be inspected first and should not show any clumping or loss of motility.

9. Examine the 1:40 and 1:80 dilutions for loss of motility and agglutination (Fig. 294).
10. Higher dilutions may be employed, but the above are ordinarily sufficient.

### AGGLUTINATION TESTS FOR TYPHOID FEVER

1. The test may be conducted microscopically with a living, smooth, agglutinable culture of the typhoid bacillus. Normal sera may agglutinate in final dilutions of 1:20 to 1:30. Agglutination at 1:40 is suspicious and at 1:80 definitely indicative of typhoid fever, providing the patient has not been immunized with typhoid-paratyphoid vaccine.

2. The test is better conducted with H (formalized) and O (alcoholic) antigens according to the slow macroscopic technic (page 627). They should be prepared of suitable strains like H901 and O901, respectively, grown on veal agar (pH 7.4).

3. Normal sera may agglutinate H antigen at about 1:20 and O antigen in final dilutions up to about 1:80.

4. Agglutination of H antigen at 1:40 is suspicious and at 1:80 or higher definitely indicative of typhoid fever in individuals who have not been previously immunized with typhoid-paratyphoid vaccine.

5. Agglutination of O antigen at 1:160 or higher is also indicative of typhoid fever in individuals who have not been immunized with typhoid-paratyphoid vaccine.

6. Active immunization with typhoid-paratyphoid vaccine produces both H and O agglutinins. The presence of O agglutinin in high titer, with H agglutinin in low titer, is indicative of typhoid fever in a previously vaccinated individual. However, a very high titer of H agglutinin as, for example, 1:1280 or 1:2560, may be indicative of typhoid fever in a previously vaccinated individual since it is rather unusual for H agglutinin to persist in titers of more than 1:640 for longer than 6 months after immunization. Consequently, when the disease is suspected in a previously vaccinated individual the tests should be repeated every 3 to 5 days with the same antigens. If the titers progressively increase, and especially for O antigen, typhoid fever is most likely present.

7. Acute infections like influenza, brucellosis, etc., in individuals previously immunized with typhoid-paratyphoid vaccine may produce a high titer of H agglutinin with a low titer of O agglutinin (*anamnesic reaction*).

8. In vaccinated individuals a drop of H, and especially of O, agglutinins to normal levels may be indicative of the advisability of re-immunization with typhoid-paratyphoid vaccine.

9. An increase of H, and especially of O, agglutinins is also of aid in the detection of typhoid carriers among nonvaccinated individuals. To be significant, the titer of H agglutinin must be above 1:20 and O agglutination above 1:100.

10. Because of the close relationship of the typhoid bacillus to the paratyphoid bacilli A and B, and especially B (*Salmonella schottmülleri*), agglutinins for the latter may be also increased in typhoid fever. However, the titer is usually considerably lower for the paratyphoid bacilli than for the typhoid bacillus. In case of doubt, the absorption test should be conducted.

**Absorption Test for Differentiating Between Typhoid and Paratyphoid Fevers.**—1. Arrange 4 rows of 4 small test tubes, each row to contain 1 cc. of serum dilutions 1:20, 1:40, 1:80 and 1:160, respectively.

2. In each tube of the first and second rows emulsify 5 large loopfuls of typhoid bacilli. Add an additional control tube carrying 1 cc. of saline and culture.

3. In each tube of the third and fourth rows place paratyphoid bacilli (A or B as decided); add a control tube.

4. Mix gently and place in water bath at 37° C. for 2 hours. The results are then recorded.

5. Centrifuge all tubes except controls and transfer supernatant fluids to 4 more rows of tubes.

6. To each tube of the first and third rows add typhoid bacilli; to the second and fourth rows add paratyphoid bacilli. Mix well and place in water bath 2 hours at 37° C.

7. If typhoid fever is present, agglutination will be strong in the first and second rows of the first part of test and practically unchanged in the third row of the second part.

8. If paratyphoid is present, agglutination will be strong in the third and fourth rows in first part of the test and practically unchanged in the fourth row of the second part. If paratyphoid B is employed with negative results, repeat the test with paratyphoid A.

### AGGLUTINATION TESTS FOR PARATYPHOID FEVER AND OTHER SALMONELLA INFECTIONS

1. As previously discussed on pages 474 to 475, a very large number of Salmonella are capable of producing gastro-intestinal infections. When these are characterized by continuous fever resembling mild typhoid fever, the infection is usually designated as "paratyphoid fever". Otherwise, only a gastro-enteritis without prolonged fever may be produced but it is often difficult to draw a sharp distinction.

2. Paratyphoid fever is usually due to *S. paratyphi* (*B. paratyphosus* A), *S. schottmülleri* (*B. paratyphosus* B) or *S. paratyphi* C (*S. hirschfeldii*) and especially *S. schottmülleri*. Among the most frequent causes of other Salmonella infections producing gastro-enteritis are *S. enteritidis* (*B. enteritidis*), *S. aertryche* (*B. typhimurium*), *S. cholera-suis* (*B. suispestifer*), *S. newport*, *S. kentucky*, etc.

3. Agglutination tests with patient's serum for paratyphoid fever are usually conducted with antigens of *S. paratyphi*, *S. schottmülleri* and *S. paratyphi* C.

4. The tests may be conducted microscopically with living cultures as described above with the typhoid bacillus. Normal sera may agglutinate in final dilutions up to 1:20 or 1:30. Agglutination at 1:40 is suspicious and at 1:40 or higher diagnostic in the case of individuals who have not been immunized with typhoid-paratyphoid vaccine.

5. The tests are better conducted, however, according to the slow macroscopic technic (page 000), with H and O antigens prepared of selected smooth strains cultivated on veal agar (pH 7.4). Some laboratories use only H antigens.

6. Normal sera may agglutinate the H antigens at about 1:20 and the O antigens in final dilutions up to about 1:80.

7. Agglutination of an H antigen at 1:40 is suspicious and at 1:80 or higher definitely indicative of paratyphoid fever in individuals who have not been previously

immunized with typhoid-paratyphoid vaccine. The same is true of agglutination of an O antigen at 1:160 or higher under these conditions. Cross-agglutination reactions with H and O antigens of the various *Salmonella* are commonly observed, but as a general rule, the titers will be highest for both the H and O antigens of the particular *Salmonella* producing infection, especially the H antigen. Previously immunization with typhoid-paratyphoid vaccine, anamnestic reactions and the carrier state influence the reactions in the same manner as described above in agglutination reactions in typhoid fever.

8. In gastro-enteritis due to *Salmonella* infections, agglutination tests conducted with patients' sera are not usually of diagnostic value. The tests, however, are highly valuable in the final identification of *Salmonella* recovered in cultures of the stools, foods, etc. These tests are best conducted with rabbit immune sera from which the group agglutinins have been removed by absorption. Cultures of the living bacilli may be employed as antigens in microscopic tests; otherwise, H antigens are preferred for the conduct of macroscopic slow agglutination tests.

### AGGLUTINATION TEST FOR BACILLARY DYSENTERY

1. Agglutination tests are of little or no value in the diagnosis of acute bacillary dysentery, but may be of diagnostic aid in chronic cases of the disease.

2. Formalized antigens of the Shiga, Flexner, New Castle and Sonne strains of dysentery bacilli and the slow macroscopic agglutination test are recommended.

3. Positive reactions in final dilution of serum 1:40 or higher with an antigen of *Bacterium shigae* (*Shigella dysenteriae*) are of diagnostic import. With antigens of *Bacterium flexneri* (*Shigella paradyenteriae*), *Bacterium sonnei* (*Shigella sonnei*) and the New Castle strain, positive reactions in final dilutions of serum 1:160 or higher may be regarded as diagnostic.

### AGGLUTINATION AND OPSONOCYTOPHAGIC TESTS FOR BRUCELLOSIS

1. Brucellosis may be due to infection with *Brucella abortus*, *Brucella melitensis* or *Brucella suis*, as discussed on pages 459 to 460. Most cases in the United States are due to *Brucella abortus*. The disease may be mistaken for typhoid fever. It is therefore a growing and praiseworthy custom to routinely conduct an agglutination test with *Br. abortus* antigen with all sera submitted for the typhoid and paratyphoid agglutination tests.

2. The rapid slide or macroscopic slow methods may be employed. Formalized or H antigens for the respective methods are preferred, but phenolized antigens, as well as heat-killed antigens preserved with formalin, are suitable. *Brucella abortus* antigen should be prepared of strain No. 456 (National Institute of Health). Since this strain appears to be antigenically sufficient the tests may be conducted with antigens prepared of this single culture. Some laboratories prefer to use routinely a separate antigen prepared of *Br. melitensis* at the same time, while others use a mixed antigen prepared of both *Br. abortus* and *Br. melitensis*. The brucella may be cultivated on 1 per cent dextrose veal agar (pH 6.8) or on liver infusion agar.



3. Blood for the agglutination and opsonocytophagic tests should be collected before the brucellergen skin test is conducted.

4. About 98 per cent of the sera of normal individuals with *negative skin reactions* do not agglutinate in dilutions higher than 1:10. Agglutination at 1:30 to 1:50 or higher constitute a positive reaction under these circumstances.

5. About 30 per cent of the sera of normal individuals with *positive skin reactions* may agglutinate in dilutions up to 1:100. Consequently, agglutination in dilutions higher than 1:100 is usually required for a positive reaction in this group.

6. Positive agglutination reactions are usually indicative of brucellosis and especially if they become progressively stronger when repeated at intervals. Positive reactions, however, may be due to previous unrecognized infections with *Brucella* and result in diagnostic errors when occurring in individuals with other illnesses.

7. Negative agglutination reactions alone or with negative skin and opsonocytophagic reactions do not necessarily exclude brucellosis.

8. As shown by Calder (*Jour. Bacteriol.*, 41: 593, 1941) agglutinin for *B. proteus* (OX19) may be increased in brucellosis, probably due to certain antigenic components shared by the respective somatic antigens or because *Proteus* is more easily agglutinated than *Brucella*. However, agglutinin for *Proteus* does not progressively increase in brucellosis as in typhus fever and other rickettsial diseases. Agglutinin for *Pasteurella tularensis* is seldom produced and the titer is usually low.

**Opsonocytophagic Test.**—This test, modified after that of Huddleson, Johnson and Hamann (*Am. Jour. Pub. Health*, 23: 917, 1933) may be conducted as follows:

1. Prepare a suspension of *Brucella* by washing off cultures on a solid medium with 0.4 per cent commercial formalin in normal saline solution. After standing for 24 hours at room temperature, centrifuge, discard the supernatant fluid and suspend the sediment in sufficient 0.1 per cent formalin in normal saline solution to give a density of such degree that a wire loop can just be seen 2 cm. below the surface of the suspension in a test tube (Gates).

2. Collect 2.0 cc. of blood in a small test tube containing 0.1 cc. of a 10 per cent solution of sodium citrate in normal saline solution. Mix to prevent coagulation and place in refrigerator.

3. Within 4 hours place 0.1 cc. of citrated blood and 0.1 cc. of bacterial suspension in a small sterile test tube. Mix thoroughly and place in a water bath at 37° C. for 30 minutes. Mix thoroughly, remove a small amount with a capillary pipet and prepare a thin smear in the same manner as for a differential leukocyte count.

4. Dry quickly to prevent shrinkage of the leukocytes, pass the slide through a Bunsen flame four times and allow to cool. Cover the smear with a 1:10 dilution of Ziehl-Neelsen carbolfuchsin and stain for 2 minutes; wash gently with tap water and allow to dry (Bondi). *Brucella* and the nuclei of phagocytes stain a deep red; the cytoplasm is a light pink and the granules are not stained. Phagocytized *Brucella* are easily counted.

5. The average number of *Brucella* engulfed by 25 leukocytes is determined. The absence of phagocytosis constitutes a negative reaction, from 1 to 20 *Brucella* per cell slightly positive, 21 to 40 moderately positive, and over 40 strongly positive.

6. About 70 per cent of normal individuals with negative skin reactions give negative opsonocytophagic reactions. About 98 per cent with positive skin reactions show positive opsonocytophagic reactions due to previous unrecognized infections with

*Brucella*. Positive skin and agglutination reactions, along with a negative or weakly positive opsonocytophagic reaction are usually indicative of brucellosis.

### AGGLUTINATION AND OPSONOCYTOPHAGIC TESTS FOR TULAREMIA

1. Formalized antigen of *Pasteurella tularensis* (No. 38) and the macroscopic slow agglutination test are preferred. The micro-organism grows slowly; blood glucose cystine agar is recommended.

2. Agglutination at 1:80 or higher constitutes a positive reaction, but lower titers may be significant if there has been a previous negative reaction, or if positive skin and opsonocytophagic reactions are observed. Positive reactions do not usually occur until the second week of the disease so that, in suspected tularemia several tests may be required before diagnosis is established or the disease excluded.

3. Cross agglutination with *Br. abortus* and *B. proteus* (OX19) may occur, but usually only in lower dilutions of serum. As in brucellosis, the acquired agglutinin tends to persist in the serum for years and even for the balance of life.

4. The *opsonocytophagic test* is conducted with a formalin-killed suspension of the bacterium in the same way as the opsonocytophagic test with *Brucella*. Positive reactions usually develop between the second and third weeks of the disease, but their interpretation is quite difficult in some cases since many weakly positive, and occasionally even strongly positive, reactions have been observed in conditions other than tularemia. The test, however, may be of aid in conjunction with skin and agglutination tests in questionable cases of tularemia and particularly when there is cross agglutination between *P. tularensis* and *Br. abortus*.

### AGGLUTINATION TESTS FOR TYPHUS FEVER, ROCKY MOUNTAIN SPOTTED FEVER AND OTHER RICKETTSIAL DISEASES

1. The Weil-Felix agglutination test has proven of value in the diagnosis of the different types of typhus fever, Rocky Mountain spotted fever and other rickettsial diseases but is of limited value in differentiating among them.

2. Formalized antigens of the OX19 (National Institute of Health No. 504), OX<sub>2</sub> and OXK strains of *B. proteus* are commonly employed in the macroscopic slow agglutination test. Some laboratories prefer alcoholic antigens of these strains. Proper controls, consisting of a known positive human or rabbit immune serum and a known normal human serum, should always be tested at the same time.

3. Normal human sera do not ordinarily agglutinate antigens of these strains in final dilutions higher than 1:25 to 1:50.

4. As a rule, in most typhus fever patients the agglutinin titer is 1:25 on the fourth day and 1:50 or higher by the eighth day. By the end of the second week it usually climbs to several thousands (even as high as 1:50,000), after which it declines rapidly during convalescence. The reaction may become negative within 5 or more months after recovery or may persist for several years.

5. Agglutination at 1:160 or higher usually occurs in Rocky Mountain spotted fever as, likewise, in the eastern type of the disease. According to Baker (*Ann. Int. Med.*, 17: 247, 1942) a test should be conducted as soon as the disease is suspected or when the eruption first appears, a second test on or about the twelfth day and a

third during convalescence. A positive reaction is indicated by an increasing titer of agglutination.

6. In the rickettsial diseases cross agglutination reactions may occur with antigens of *Br. abortus*.

7. The following table, after that by Felix (*Brit. Med. Jour.*, Nov. 21, 1942), may be helpful in the differentiation of typhus fever, typhus-like diseases and Rocky Mountain spotted fever:

Disease	Agglutination *		
	OX19	OX <sub>2</sub>	OXK
Epidemic typhus	+++	+	—
Tabardillo (Mexico)	+++	+	—
Brill's disease (U.S.A.)	+++	+	—
Tsutsugamushi fever	—	—	+++
Scrub typhus	—	—	+++
Sao Paulo typhus	+	+	+
Fièvre boutonneuse	+	+	+
Tick bite fever (South Africa)	+	+	+
Tick born typhus of India, Kenya, etc.	+	+	+
Rocky Mountain spotted fever	+	+	+

\* + + + = main agglutination; + = group agglutination; — = negative.

### AGGLUTINATION TESTS FOR LEPTOSPIRAL JAUNDICE

1. Leptospiral jaundice (infectious jaundice or Weil's disease) is usually due to infection with *Leptospira icterohaemorrhagiae* and sometimes to *Leptospira canicola*. *Leptospira grippotyphosa* causes "swamp fever" or "harvest fever" while *Leptospira hebdomadis* produces "seven-day fever" in Japan, but these have not been reported so far in the United States.

2. A microscopic agglutination test employing living cultures of *L. icterohaemorrhagiae* has been described by Fletcher (*Tr. Roy. Soc. Trop. Med. and Hyg.*, 21: 1256, 1928) in which 0.2 cc. of varying dilutions of serum are mixed in small test tubes with 0.2 cc. of culture, incubated in a water bath at 37° C. for 1½ hours, and examined by darkfield illumination for the agglutination of leptospira. This method is very sensitive, but subject to technical error in the reading of reactions. Normal sera may show agglutination in final dilutions of 1:10 to as high as about 1:200.

3. Formalized antigens of leptospira secured by high speed centrifugation of cultures have been employed by Brown (*Brit. Med. Jour.* 2: 1183, 1939) and by Starbuck and Ward (*Jour. Infect. Dis.* 70: 88, 1942) in the rapid slide agglutination test. The reactions are not as sensitive as those observed with the microscopic test, but are less subject to error and consequently more specific while sufficiently sensitive to be of value in the diagnosis of acute cases of leptospiral jaundice.

4. With the rapid slide method normal human sera may agglutinate in final dilutions up to about 1:40. Immune agglutinins are stated to appear about the fifth day of illness with the production of weakly positive reactions (1:100). Subsequently, they rapidly increase, reaching a maximum by about the fifteenth day. Strongly

positive reactions continue to occur for about 7 weeks, after which the agglutinins decrease but usually persist for several years after recovery. However, agglutinins may not develop in some mild cases or increase to but a slight degree and then disappear within a few weeks.

### AGGLUTINATION TEST FOR PLAGUE

1. Phenolized or formalized antigens of *Pasteurella pestis* (*B. pestis*) are used in the macroscopic slow agglutination test. There is a strong tendency to spontaneous agglutination.

2. Normal sera may agglutinate in final dilutions up to about 1:10.

3. Immune agglutinins do not usually appear until about the ninth day of the disease, thus rendering the agglutination test of little value in early diagnosis. Agglutination in final dilutions of serum 1:20 to 1:40 or higher, however, is of confirmatory value in the convalescent stage or after recovery.

### AGGLUTINATION TEST FOR PERTUSSIS

1. Formalized antigens of *Hemophilus pertussis* (*B. pertussis*) are employed in the macroscopic slow agglutination test. The antigen should be prepared of an agglutinable strain of the bacillus in phase I.

2. Normal sera may give positive reactions in final dilutions up to 1:100.

3. Positive agglutination in final dilutions of 1:200 or higher may occur in the paroxysmal or later stages of the disease. The test, therefore, is of no value in diagnosis during the early stage of pertussis. It is frequently employed as a measure of antibody production following active immunization with pertussis vaccine.

### AGGLUTINATION TEST FOR GLANDERS OF MAN, HORSES AND MULES

1. Heat-killed antigen prepared of a suitable agglutinable strain of *B. mallei* and preserved with formalin in final concentration of 0.25 per cent is employed in the macroscopic slow agglutination test. The final dilutions of serum in the latter should be 1:100, 1:200, 1:400, 1:500, 1:800, 1:1000, etc.

2. Normal human sera may agglutinate in final dilutions up to about 1:100. Normal horse and mule sera may give partial agglutination in final dilutions of serum up to 1:200 to 1:400.

3. In acute glanders of man, agglutination may occur at 1:1000 or higher; in chronic glanders the titer is usually very low and may fall within the normal range.

4. In horses and mules agglutination at 1:800 is suspicious, and, at 1:1000 or higher, diagnostic.

**AGGLUTINATION TEST FOR BACILLARY WHITE DIARRHEA  
OF CHICKENS**

1. Phenolized antigen of a suitable agglutinable strain of *Salmonella pullorum* is employed in the macroscopic slow agglutination test. To each 100 cc. of antigen add 2 cc. of 2 per cent solution of sodium hydroxide, as recommended by Matthews for the prevention of precipitation, giving the cloudy reactions which may occur with as high as 75 per cent of sera.
2. Normal sera may give partial agglutination in final dilutions up to about 1:10.
3. Agglutination at 1:20 is suspicious and, at 1:40 or higher, diagnostic.

**AGGLUTINATION TEST FOR FOWL TYPHOID**

1. Phenolized antigen of a suitable agglutinable strain of *Salmonella gallinarum* is employed in the macroscopic agglutination test. To each 100 cc. of antigen add 2 cc. of 2 per cent solution of sodium hydroxide for aid in preventing the precipitation frequently producing cloudy reactions in tests with fowl sera.
2. Normal sera may give partial agglutination in final dilutions up to about 1:20.
3. Partial agglutination in final dilutions of 1:40 to 1:80 but not higher is suspicious; agglutination at 1:160 or higher is diagnostic.

# METHODS FOR CONDUCTING HEMAGGLUTINATION AND BLOOD GROUPING TESTS

## DAVIDSOHN PRESUMPTIVE TEST FOR INFECTIOUS MONONUCLEOSIS

**Principles.**—Heterophile agglutinin for sheep corpuscles is usually and characteristically produced in infectious mononucleosis.

Heterophile agglutinin is also produced by injections of horse serum and by various bacteria; it may be distinguished from the antibody occurring in infectious mononucleosis by differential absorption tests employing guinea-pig and rabbit kidney.

**Method.**—1. Prepare a 2 per cent suspension of washed sheep corpuscles in normal saline solution. The sheep blood should be not less than 24 hours old and not older than about 1 week, because older cells tend to become too easily agglutinable. The suspension must be prepared from cells washed on the day of the test.

2. Inactivate the patient's serum by heating in a water bath at 56° C. for 30 minutes.

3. Arrange a series of 12 small test tubes. Place 0.4 cc. normal saline solution in No. 1 and 0.25 cc. in each of the remaining tubes (Table 26).

4. Add 0.1 cc. of serum to No. 1. Mix and transfer 0.25 cc. to No. 2; mix and transfer 0.25 cc. to No. 3 and so on to No. 11 from which 0.25 cc. is discarded after mixing. No. 12 is the corpuscle control.

5. Add 0.1 cc. of 2 per cent suspension of washed sheep corpuscles to all tubes.

6. Mix all tubes and leave at room temperature for 2 hours.

**TABLE 26.—TECHNIC OF PRESUMPTIVE TEST FOR INFECTIOUS MONONUCLEOSIS**

Tubes	Saline Solution cc.	Serum cc.	Sheep Corpuscles cc.	Final Dilutions of Serum	Mix thoroughly and keep at room temperature for 2 hours; make readings.
1	0.4	0.1	0.1	1:7	
2	0.25	0.25 of 1:5	0.1	1:14	
3	0.25	0.25 of 1:10	0.1	1:28	
4	0.25	0.25 of 1:20	0.1	1:56	
5	0.25	0.25 of 1:40	0.1	1:112	
6	0.25	0.25 of 1:80	0.1	1:224	
7	0.25	0.25 of 1:160	0.1	1:448	
8	0.25	0.25 of 1:320	0.1	1:896	
9	0.25	0.25 of 1:640	0.1	1:1792	
10	0.25	0.25 of 1:1280	0.1	1:3584	
11	0.25	0.25 of 1:2560	0.1	1:7168	
12	0.25	control	0.1	—	

7. Read the results after gently shaking the tubes. The tubes in which the cells remain in the form of a single large clump are read as +++. Those in which the cells break up into distinctly visible clumps and the fluid is clear and transparent are read as ++. The reading of + agglutination is best determined by holding the tube horizontally on the stage of a microscope and examining with the low power objective. With experience the reading can be made macroscopically but is usually 1 or 2 dilutions lower than the titer determined by microscopic examination. When time

permits, it is advisable to repeat the reading after an overnight incubation in the refrigerator. The titer of the serum is then usually 1 or 2 dilutions higher. The corpuscle control should show no agglutination.

**Interpretation.**—In the absence of recent injections of horse serum and serum sickness, the titer is not higher than 1:7 under normal conditions. Agglutination at 1:14 to 1:28 is suggestive of infectious mononucleosis; about 90 per cent of cases show agglutination at 1:28 or higher. Agglutination in final dilutions of serum 1:224 or higher is diagnostic of infectious mononucleosis providing serum sickness can be excluded even though injections of horse serum have been given. Positive reactions do not occur in the leukemias, Hodgkin's disease, etc.

### DAVIDSOHN DIFFERENTIAL TEST FOR INFECTIOUS MONONUCLEOSIS

**Principles.**—The heterophilic antibodies (antisheep agglutinin) in infectious mononucleosis are not of the Forssman type. They are not absorbed by a suspension of guinea-pig kidney. The heterophilic antibodies in serum disease are of the Forssman type and are readily absorbed by a suspension of guinea-pig kidney.

The antisheep agglutinins are absorbed by boiled beef red corpuscles from the sera of patients with infectious mononucleosis and with serum disease, but not from normal sera.

The test is indicated when the patient has serum disease or has had a recent injection of horse serum when the titer is 1:224 or higher in the presumptive test. It is also sometimes advisable when the titer is 1:56 or 1:112 in the presumptive test under these conditions.

**Preparation of Reagents.**—1. *Guinea-Pig Kidney.*—The kidneys of the guinea-pig are kept frozen in the refrigerator until needed. They are then thawed and washed repeatedly in a physiological solution of sodium chloride until the washings are free of blood. They are now mashed into a fine pulp and used for absorption as a 20 per cent suspension in physiological salt solution. The suspensions are boiled for 1 hour on the water bath and the loss by evaporation made up with distilled water.

2. *Beef Cells.* The beef red cells are washed 3 times, packed well in the centrifuge, suspended in 4 volumes of a physiological salt solution and boiled for 1 hour on the water bath. The loss by evaporation is made up with distilled water.

Enough phenol is added to the antigenic suspensions to make a 0.5 per cent solution. The antigens may be kept in the ice-box for many months without a noticeable change.

**Absorption with Boiled Guinea-Pig Kidney Antigen.**—1. Place in a test tube ( $85 \times 13$  mm.) 0.5 cc. of the thoroughly shaken 20 per cent suspension of boiled guinea-pig kidney.

2. Add 0.1 cc. of serum that has been heated for 30 minutes at  $56^{\circ}$  C.

3. Shake and let stand at room temperature for 1 hour, shaking at 15-minute intervals.

4. Centrifuge at 1500 revolutions for 10 minutes.

5. Remove the supernatant fluid with a capillary pipet.

6. To a row of 6 tubes ( $75 \times 12$  mm.) add 0.25 cc. of physiological salt solution.

7. To the first tube add 0.25 cc. of the absorbed serum.

8. Mix and transfer 0.25 cc. to the second tube, etc. Discard 0.25 cc. from the last tube. The serum dilutions are: 1:10, 1:20, 1:40, etc.

9. Add 0.1 cc. of a 2 per cent suspension of sheep cells. Shake well. Final dilutions of serum are 1:14, 1:28, etc. Let stand at room temperature for 2 hours. Read.

**Absorption with Boiled Beef Corpuscle Antigen.**—Exactly the same procedure as above, using 0.5 cc. of the thoroughly shaken 20 per cent suspension. If it is necessary to begin with dilution of 1:7 as may be the case in serums with titers below 1:112, then add 0.2 cc. of serum to 1 cc. of the antigen suspension. For titration, omit the physiological salt solution from the first tube, but in the other tubes, place the usual amount of 0.25 cc. From the absorbed serum, add 0.25 cc. for the first and to the second tube. Proceed as above. Final dilutions are: 1:7, 1:14, etc.

**Control with Unabsorbed Serum.**—At the same time carry out a diagnostic test on unabsorbed serum according to the previously outlined technic to have a basis for comparison.

**Interpretation.**—In the case of infectious mononucleosis, the absorption of the serum with the suspension of the guinea-pig kidney will effect a partial removal of the agglutinins for sheep red cells, but not less than one-fourth of the titer will remain: f. i. the titer before absorption 1:112, after the absorption with the guinea-pig kidney 1:28. If all or almost all of the agglutinins were removed then this speaks against infectious mononucleosis. The absorption with ox cells is a confirmatory procedure. The agglutinins for sheep red cells are completely or almost completely removed by beef cells.

## MAJOR BLOOD GROUPS

The plasma of a patient (recipient) may agglutinate or hemolyze the corpuscles of a donor or the corpuscles of the recipient may be agglutinated or hemolyzed by the plasma of a donor. Blood transfusion requires, therefore, that the blood of a donor be compatible with that of the patient. Hemolysis does not occur without agglutination and since the latter is more easily detected, it is sufficient to test only for agglutination.

1. According to Landsteiner, there are 4 major blood groups designated according to the International Nomenclature as O, A, B and AB. These depend upon the presence or absence of 2 agglutinogens A and B in the erythrocytes and 2 specific agglutinins, *a* (or anti-A) and *b* (or anti-B) in the serum. If the corpuscles contain neither agglutinin the serum contains both agglutinins and the blood belongs to group O (so-called universal donor). If the corpuscles contain agglutinin A, the serum contains agglutinin *b* and the blood belongs to group A. If the corpuscles contain agglutinin B, the serum contains agglutinin *a* and the blood belongs to group B.

If the corpuscles contain both agglutinogens A and B, the serum is free of the agglutinins *a* and *b* and the blood belongs to group AB. In other words, the serum regularly contains the agglutinins for the absent agglutinogens; that is, corresponding agglutinins and agglutinogens do not coexist in the blood of an individual.

2. The 4 major blood groups are also classified according to the numberings of Moss and Jansky as shown in Table 27. It is to be noted especially that group I of Moss corresponds to IV of Jansky and that IV of Moss corresponds to I of Jansky. If blood grouping is reported by numbers, it is essential therefore always to state whether the Moss or Jansky classification is being used, in order to avoid serious errors and accidents in blood transfusions. Under the conditions it is advisable to employ



exclusively the Landsteiner or International Nomenclature. The percentages of the 4 major blood groups vary according to race and in any particular race are modified to some extent by disease; according to Wiener, those shown in Table 27 are fairly representative of healthy individuals in the United States.

TABLE 27.—MAJOR BLOOD GROUPS

International Nomenclature	Moss	Jansky	Agglutininogen in Corpuscles	Agglutinin in Serum or Plasma	Percentages
O	IV	I	—	<i>ab</i>	45
A	II	II	A	<i>b</i>	39
B	III	III	B	<i>a</i>	12
AB	I	IV	AB	—	4

3. As a rule agglutinogens are demonstrable in the corpuscles of the newborn but for a year or two are of low sensitivity to agglutination. Agglutinogens absent at birth do not appear later on in life. But only about half of all newborn infants have demonstrable agglutinins in their sera; whatever agglutinins are present at birth are thought to be derived from the mother by filtration through the placenta. They are gradually acquired after birth but may not reach the proportions of adults until 2 or more years of age.

### BLOOD SUBGROUPS

1. Agglutininogen A also exists in subgroup  $A_1$ ,  $A_2$  and  $A_3$ .  $A_2$  makes up approximately  $\frac{1}{4}$  to  $\frac{1}{6}$  of all group A individuals. Since  $A_2$  cells are not usually as susceptible to agglutination by B serum as A and  $A_1$  cells, it is clear that with a weak group B serum 20 to 25 per cent of group A individuals may be erroneously classified as group O.

Under the conditions group AB also contains the 3 subgroups  $A_1B$ ,  $A_2B$  and  $A_3B$ .  $A_1B$  is about one and one-half times as common as  $A_2B$ . In group AB errors in grouping are even more likely to occur,  $A_2B$  blood often being erroneously classified as group B.

2. Agglutinins for these subgroups occur only in blood belonging to group O and B. Agglutinin  $a_1$  will agglutinate  $A_1$  corpuscles but not  $A_2$  corpuscles; agglutinin  $a_2$  will agglutinate both  $A_1$  and  $A_2$  corpuscles. But these agglutinins are usually of the "cold" type and consequently their presence in the blood of donors and recipients does not usually produce reactions. In other words,  $A_1$  blood may be safely given to  $A_2$  patients, or vice versa; consequently, these subgroups are not ordinarily considered in pre-transfusion blood tests. In those cases where reactions have occurred Wiener has ascribed them to the presence of Rh agglutinin in the plasma of donors transfused with corpuscles containing Rh agglutininogen. But these subgroup agglutinins may sometimes agglutinate at  $15^\circ$  to  $20^\circ$  C. and thereby lead to confusion and error in pre-transfusion and agglutination tests conducted at room temperature. Furthermore, these subgroup agglutinins may be increased by multiple transfusions and especially with the blood of the same donor. They may also be produced during pregnancy through isoimmunization by the fetus. Under these circumstances they may agglutinate at body temperature ("atypical warm agglutinins") and their presence in the blood of

recipients may produce reactions. Under the circumstances it is advisable to select compatible donors whose blood does not contain these subgroup agglutinins in the case of patients receiving multiple transfusions and for the transfusion of women during pregnancy and the puerperium.

3. Human corpuscles may also contain the agglutinogens M, N and P. Agglutinins for them, however, are but rarely found in normal blood. Furthermore, these agglutinogens are of very low antigenicity and agglutinins for them are not usually produced by multiple transfusions or during pregnancy. Under the circumstances these agglutinogens and agglutinins are not routinely examined for in pre-transfusion blood tests. Examinations for M and N agglutinogens, however, are very useful in connection with tests for the exclusion of paternity.

4. The corpuscles of about 85 per cent of human beings also contain the Rh agglutinin (Rh+) discovered by Landsteiner and Wiener (*Proc. Soc. Exper. Biol. and Med.* 43: 223, 1940). Agglutinin for it does not occur normally in the blood. Consequently, Rh+ corpuscles do not produce reactions in first transfusions. However, the agglutinin is antigenic and multiple transfusions with Rh+ corpuscles may produce an Rh agglutinin. The latter may be also produced during pregnancy through iso-immunization by Rh agglutinin in the fetus. The agglutinin is also of the "cold" type but the presence of large amounts in the blood of recipients may produce reactions upon transfusion with Rh+ corpuscles; probably it is also responsible for erythroblastosis fetalis. Consequently, it is advisable to select compatible donors whose corpuscles are Rh— for the transfusion of individuals who have had previous transfusions (especially with Rh+ corpuscles) as likewise for the first and subsequent transfusions of women in pregnancy and the puerperium.

5. Bloods which lack their full complement of iso-agglutinogens and iso-agglutinins are known as *defective blood groups*. Thus, individuals of group A may show no agglutinins in the serum (A, o) or group O with only a agglutinin (O, a). Although the occurrence of this anomaly is very frequent in newborn infants, it rarely occurs in adults and most of the cases which have been reported were probably due to weak reactions which were overlooked.

## PRECAUTIONS IN PRETRANSFUSION BLOOD TESTS

1. Although pretransfusion blood tests are in themselves relatively simple procedures, their performance requires a high degree of individual and group responsibility. In each laboratory where these tests are made there should be a standard routine procedure designed especially to eliminate mistakes and prevent accidents. Extreme care should be exerted to avoid the mixing up of specimens, etc. All test tubes and slides should be labeled, the tubes containing serum and cell suspensions of the same individual should be kept adjacent; recipient's blood may be kept in slightly longer tubes.

2. For blood transfusion the recipient's blood should be typed and a donor selected belonging to the same group. But direct matching is recommended in addition, due to the existence not alone of subgroups and atypical agglutinins, but also of donors with unusually high agglutinin titers. Such high agglutinin titers may be dangerous when a so-called universal donor O (I Jansky, IV Moss) is used. However, it is not quite safe to depend on direct matching alone since in cases of low titer agglutinins,

incompatibility may escape attention. In other words, if only one of the two methods for selecting donors is to be depended on direct matching is preferable, but it is better and therefore recommended that the blood of the recipient be typed and direct matching tests conducted with the blood of donors belonging to the same group for the final selection of a donor.

3. Special care should be exercised in testing the blood of infants under 2 years of age, due to uncertainty in the time of appearance of permanent iso-agglutinins.

4. Obviously only healthy donors should be chosen. Those with fever or anemia should not be used. *Syphilis and malaria should be excluded.*

5. It is always advisable to preserve blood specimens for re-checking if necessary.

### SOURCES OF ERROR IN PRETRANSFUSION BLOOD TESTS

**False Negative Reactions.**—Failure of agglutination resulting in falsely negative reactions may be due to various causes as follows:

1. The use of weak group A and group B sera containing insufficient amounts of agglutinins.

2. Undue haste in conducting the tests resulting in reading the reactions too soon after mixing corpuscles and sera.

3. Using too concentrated suspensions of corpuscles which may absorb all of the agglutinins present in weak or diluted sera, but fail to show agglutination.

4. Using corpuscle suspensions which have been kept too long with a consequent reduction or loss of sensitivity to agglutination.

5. The use of corpuscles carrying agglutinogens which are too low in sensitivity to agglutinins, as in the case of newborn infants; likewise, in the case of the fresh blood of group A adults in whom the A agglutinin is lacking in sensitivity with the result of mistaking AB blood of subgroup A<sub>2</sub> for group B.

6. The occurrence of marked hemolysis with the masking of agglutination when fresh and highly active sera are employed. The absence of clumps of erythrocytes (without regard for evidence of hemolysis) may suggest compatibility in direct matching tests. By keeping such a possibility in mind and comparing the slide with the control (cells and saline solution), the true nature of the phenomenon can easily be recognized. Hemolysis is readily detected by the increased transparency of the mixture and should be considered equivalent to agglutination. The phenomenon, which does not occur with inactivated or stored serum, is due to the presence of complement in fresh serum.

**False Positive Reactions.**—These may be due to various causes, as follows:

1. *Pseudo-agglutination*, due to rouleaux-formation (Fig. 295) in which the corpuscles occur in piles like coins, resulting from an increased viscosity of the serum of patients with an increased sedimentation rate of the corpuscles. The phenomenon is favored by using serum in too high concentration and conducting the tests at too high temperature. This source of error is readily avoided by proper dilution of corpuscles and sera.

2. *Auto-agglutination* or agglutination of an individual's erythrocytes by his own serum at room or lower temperatures, but disappearing when the mixture is warmed to 37° C. This is due to the presence of an auto-agglutinin in the serum with a corresponding agglutinin in the corpuscles. The phenomenon is particularly apt to

occur in paroxysmal hemoglobinuria, syphilitic or hypertrophic cirrhosis of the liver, hemolytic jaundice, Raynaud's disease, trypanosomiasis and severe anemias, but may result in considerable confusion because the sera may also agglutinate the corpuscles of all other human beings regardless of the blood group.

3. *Bacteriogenic agglutination* due to changes in erythrocytes and serum induced by bacterial contamination and consequently given this designation by Davidsohn and Toharsky (*Jour. Inf. Dis.* 67: 25; 1940; *Jour. Immunol.* 43: 213, 1942). The reaction was formerly known as "panagglutination" or the "Hübener-Thomsen phenomenon" because, when due to changes in the corpuscles, the latter are agglutinated by any normal human serum, including that of the patient from whom blood had been obtained. The reaction is likely to occur in tests on menstrual and post-mortem blood. It may be avoided by using fresh corpuscles and group A and group B sera protected against bacterial contamination by suitable preservatives.

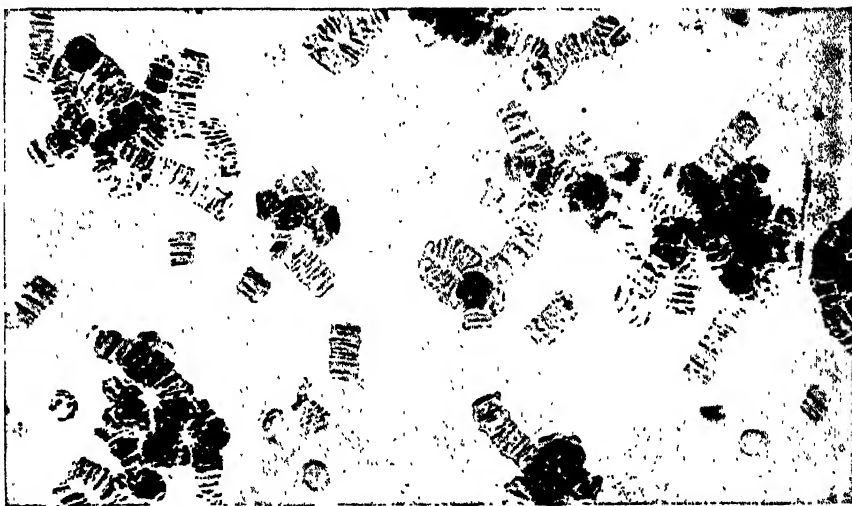


FIG. 295.—ROULEAUX FORMATION  
(From Wiener, *Blood Groups and Transfusion*, Chas. C. Thomas, Springfield, Ill.)  $\times 400$ .

4. *Irregular or atypical iso-agglutination*, due to anomalous or subgroup agglutinins in normal sera. They are usually weak and act only in the cold ("cold agglutinins") but may cause agglutination at room temperature. If the group A and B testing sera are kept in a refrigerator, care should be taken that room temperature be approximated before using them. Such agglutinins can be removed by separating serum from corpuscles at 0 to 5° C.

The most common of these atypical iso-agglutinins are (a) the anti-A<sub>1</sub> agglutinins occurring in individuals of subgroups A<sub>2</sub> and A<sub>2</sub>B. They also include (b) anti-O agglutinins reacting with all group O corpuscles and less intensely with the corpuscles of A<sub>2</sub>; (c) agglutinins specific for corpuscles containing agglutigen P and (d) unclassified irregular iso-agglutinins.

In addition to these irregular or atypical iso-agglutinins occurring in normal sera are those produced by iso-immunization from repeated blood transfusions and during pregnancy. While many of these immune iso-agglutinins are of the "cold" variety,

not infrequently they cause more agglutination at higher temperatures (best at about 37° C.) constituting the so-called "atypical warm agglutinins" of Levine.

5. *Secondary coagulation* may also give false positive reactions by simulating agglutination when unwashed corpuscle suspensions, prepared from whole blood taken directly from the finger, and fresh serum are used. It does not occur with washed corpuscles suspensions or when stored sera are employed.

6. As shown by Wiener and his colleagues, false agglutination may also occur with sera derived from *umbilical cord* specimens of blood; they are thought to be due to the presence of Wharton's jelly in the serum (*Jour. Immunol.* 17:545, 1929).

### METHODS FOR BLOOD GROUPING TESTS

**Testing Sera.**—1. Sera from individuals belonging to groups A and B are required. They should be of high titer. This is especially true of group B serum in order to detect and properly classify individuals belonging to the subgroups of A ( $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_1B$ ,  $A_2B$  and  $A_3B$ ) because the corpuscles of these subgroups are not as susceptible to agglutination as A corpuscles and especially  $A_2$  and  $A_2B$  cells. Sera from healthy young adults are preferred since those prepared from the blood of young children and aged individuals are apt to be weak in agglutinins.

2. Blood from group A and group B donors should be collected aseptically by venipuncture in sterile centrifuge or test tubes. Allow the tubes to stand in a refrigerator overnight for the absorption of any auto-agglutinins by the corpuscles. Separate the sera (if necessary by centrifugation), being careful to avoid contamination with saliva if pipets are used. Inactivate the sera by heating in a water bath at 56° C. for 30 minutes; this does not destroy agglutinins but tends to render positive reactions more definite and easier to read.

3. To each cc. of group A serum add 0.01 cc. each of 1 per cent aqueous solution of neutral acriflavine and 0.5 per cent aqueous solution of basic fuchsin; to each cc. of group B serum add 0.01 cc. of 1 per cent aqueous solution of brilliant green, as recommended by Rosenthal (*Jour. Lab. and Clin. Med.* 16: 1123, 1931). Otherwise, the sera may be preserved by adding 0.2 cc. of 1:1000 aqueous solution of merthiolate to each cubic centimeter. Each should be carefully labelled and dated. They may be dispensed in stock bottles or in 1 cc. ampules (preferred) and kept in a refrigerator but should be allowed to warm up for an hour or two at room temperature before use.

4. According to Wiener, a satisfactory group A serum should agglutinate group B corpuscles in final dilution of 1:20 and preferably 1:40. A satisfactory group B serum should agglutinate group A corpuscles in final dilution of 1:40 and preferably 1:80 in order to detect  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_1B$ ,  $A_2B$  and  $A_3B$  individuals. Titrations may be conducted as follows: (a) For each serum arrange 8 small test tubes and place 0.1, 0.4, 0.2, 0.2, 0.2, 0.2, 0.2 and 0.2 cc. saline solution respectively; (b) add 0.1 cc. serum to Nos. 1 and 2; mix No. 2, transfer 0.2 cc. to No. 3 and discard 0.1 cc.; mix No. 3 and transfer 0.2 cc. to No. 4 and so on to No. 7 from which discard 0.2 cc. after mixing (No. 8 is the corpuscle control); (c) add 0.2 cc. of a 2.0 per cent suspension of washed (once) corpuscles in saline to each tube (use group B corpuscles in titrating group A serum and group A corpuscles in titrating group B serum); (d) mix all tubes and allow to stand at room temperature for 2 hours with occasional shaking when the readings are made. The final dilutions of serum are 1:4, 1:10, 1:20, 1:40,

1:80, 1:160 and 1:320 respectively. The highest dilution showing partial but definite agglutination as compared with the corpuscle control indicates the titer.

5. Laboratories may maintain and replenish stocks of group A and group B sera by testing excess sera left over on their clots from specimens of blood submitted for the Wassermann test. Separate the sera and prepare suitable suspensions of the corpuscles in saline solution by breaking up the clots. Determine the blood group of the corpuscles from each clot by the slide method (see below) using known group A and group B sera. The sera from clots of B blood belong to group A and those from clots of A blood to group B. Titrate each serum for its agglutinin content. Pool and inactivate the satisfactory A and B sera and add a preservative to each as described above. Pillemer (*Science*, Jan. 15, 1943) has recently described a method for isolating and concentrating the agglutinins in group A and group B sera by precipitation of the antibody proteins with methanol.

**Slide Methods.**—1. Puncture a finger and collect 2 drops of blood in a small test tube carrying 2 cc. of saline solution; mix gently.

2. Divide a slide down the middle with a wax pencil. Mark the left side A and the right B.

3. Place 1 drop of A serum in the center of the A side of the slide and 1 drop of B serum in the center of the B side. One drop of each is sufficient for the test.

4. Add 1 drop of corpuscle suspension to each and mix by gently rocking the slide.

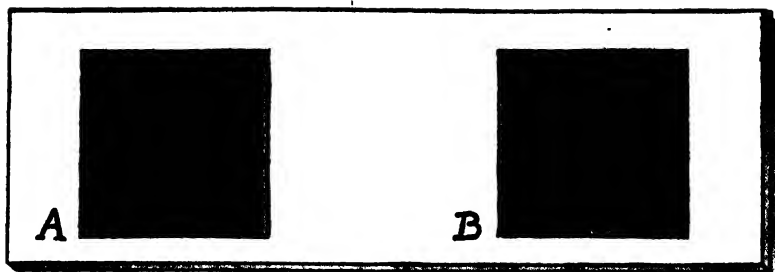
5. If blood is not collected in saline solution, transfer a minute drop obtained by puncture to the drop of A serum by means of a clean applicator and mix to a smooth suspension. With a fresh applicator, transfer a like drop to the B serum and mix thoroughly. *Never use the same applicator for both sera.*

6. Allow to stand for 3 to 5 minutes, occasionally rolling or tilting the slide to insure thorough mixing. If it is difficult to distinguish between true agglutination and rouleaux formation, stir again with the applicator, as rouleaux will be broken up to a smooth suspension thereby, but true agglutination will be unaffected. If definite agglutination has occurred at the end of 5 minutes, a reading and report may be made. If there is no definite agglutination, cover each mixture with a coverglass and examine at intervals (macroscopically and microscopically), making a final reading at the end of 30 minutes. Sometimes a final reading must be deferred for 60 minutes in the case of group AB blood; in this case it is advisable to ring the preparations with vaselin to prevent evaporation.

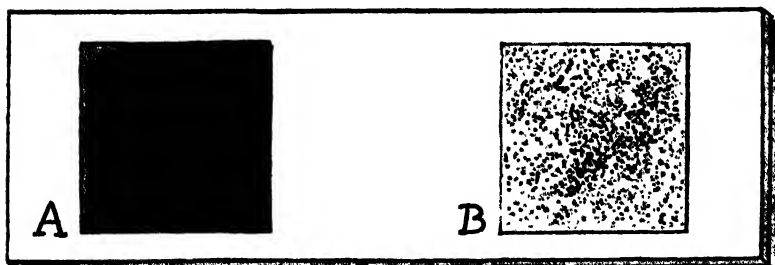
7. The readings are made as follows (Fig. 296): (a) No agglutination by either A and B sera = group O; (b) agglutination by B serum but not by A serum = group A; (c) agglutination by A serum but not by B serum = group B; (d) agglutination by both A and B sera = group AB. Tests conducted with corpuscles in saline solution are to be preferred and especially in the case of doubtful reactions; it may be necessary to examine the mixtures with low power magnification to determine the presence or absence of agglutination. A corpuscle control prepared of a drop of the suspension and a drop of saline solution is advisable.

8. A large number of tests may be conducted simultaneously by an open slide method. For this purpose the micro test slide carrying twelve wells and the electric shaking machine of Boerner, are very satisfactory; likewise the paraffin-ring slides of Kline. These are employed in conducting micro-flocculation tests for syphilis and

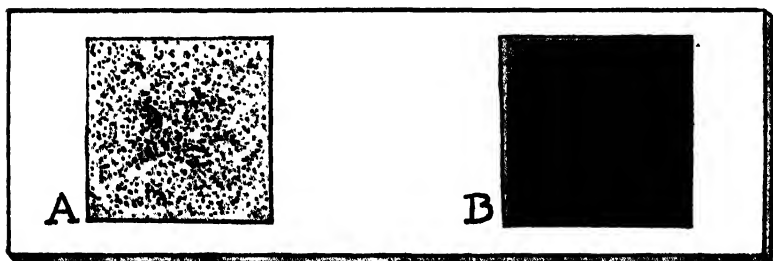
GROUP O



GROUP A



GROUP B



GROUP AB

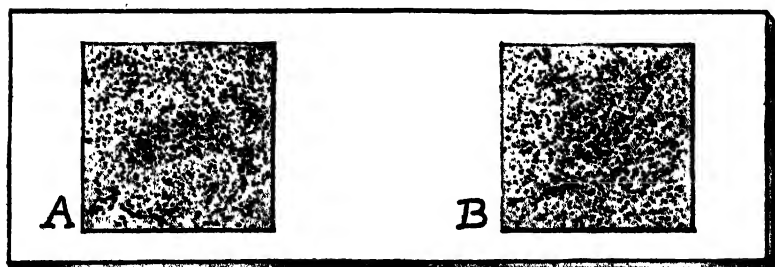


FIG. 296.—BLOOD GROUPING ACCORDING TO THE LANDSTEINER OR INTERNATIONAL CLASSIFICATION  
(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

are described on pages 739 to 740. No more than 36 tests should be attempted at one time lest evaporation interfere with the reactions. It is also advisable to use each serum diluted 1:2 or 1:3 to prevent pseudo-agglutination.

**Test Tube Method (Landsteiner).**—1. Collect 2 drops of blood in a small test tube carrying 1 or 2 cc. of saline solution.

2. In a small test tube (inside diameter 7 mm.) place 1 drop of corpuscle suspension, 1 drop of A serum and 1 drop of normal saline solution. Prepare a similar mixture in a second tube using B serum.

3. Mix gently and allow to stand at room temperature. Positive reactions are usually visible within a few minutes, but the final reading should be made after an hour. Transfer small drops of each mixture to glass slides by means of stirring rods and examine microscopically under low power. Control tests with known corpuscles of groups A and B are advisable.

4. The reactions can be hastened and intensified by centrifuging the tubes at about 2000 r.p.m. for about 1 minute. The tubes are then replaced in the rack, which is shaken until the negative control corpuscles are evenly suspended. The reactions are read macroscopically.

5. The readings and interpretations are made as described above.

## METHODS FOR CROSS MATCHING TESTS

When conditions permit, direct matching tests are always advisable before transfusion even if the patient is first grouped and a donor of the same group is selected in order to guard against the possibility of transfusing with an incompatible subgroup blood. This is particularly true in relation to subgroups  $A_1$ ,  $A_2$ ,  $A_1B$ ,  $A_2B$  and Rh, and especially in the case of patients belonging to groups A or B receiving multiple transfusions and women in pregnancy or the puerperium.

In the following tests corpuscle suspensions may be prepared by mixing 2 drops of blood with 1 or 2 cc. of saline solution in a small test tube. For serum collect blood in a dry test tube; allow to coagulate, break up the clot and centrifuge.

**Test Tube Method.**—As shown by Levine (*Jour. Obst. and Gyn.* 42: 165, 1941), these tests are best conducted in test tubes incubated at 37° C. with duplicate tests conducted at the same time at room and refrigerator temperatures, as recommended by Wiener and Peters (*Ann. Int. Med.* 13: 2306, 1940):

1. In each of 3 small test tubes mix 2 drops of patient's serum with 1 drop of donor's corpuscle suspension.

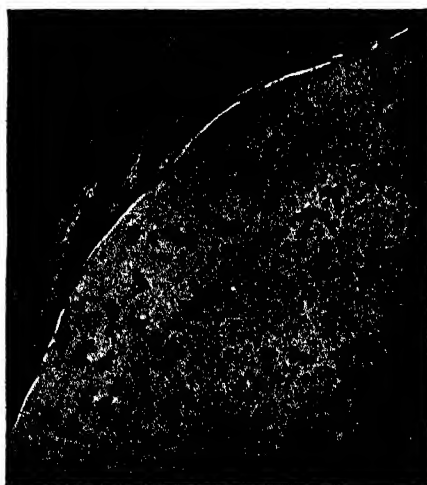


FIG. 297.—FALSE AGGLUTINATION

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)



2. Incubate 1 tube at 37° C. for ½ hour, the second at room temperature and the third in the refrigerator for 1 to 2 hours.

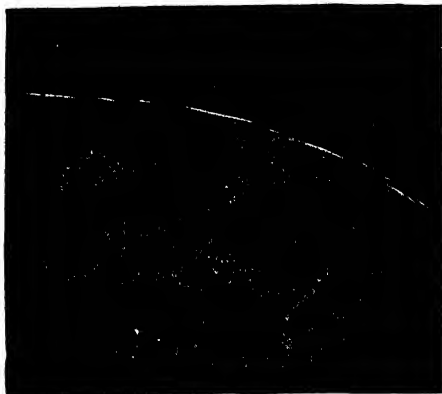


FIG. 298.—A NEGATIVE REACTION

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

with a drop of saline solution (corpuscle control).

2. At one end of slide No. 2 mix a drop of the donor's corpuscle suspension with a drop of the patient's serum and a drop of saline solution. At the other end mix a drop of the donor's corpuscles with a drop of saline solution (corpuscle control).

3. Repeat the set-up in the same manner with the corpuscles and sera of each donor.

4. Rock the slides occasionally for about 5 minutes. If agglutination is not visible, place a coverglass over each mixture and let stand for 15 minutes. Examine each microscopically.

**Hanging Drop Method.**—1. Mix 2 loopfuls of the patient's serum with 1 loopful of donor's corpuscles on a coverglass and mount with vaselin on a hanging drop slide.

2. Mix 2 loopfuls of the donor's serum with 1 loopful of patient's corpuscles on a second coverglass and mount.

3. Mix 2 loopfuls of saline solution and 1 loopful of patient's corpuscles on a coverglass and mount (corpuscle control).

4. Mix 2 loopfuls of saline solution and 1 loopful of donor's corpuscles on a coverglass and mount (corpuscle control).

3. Centrifuge the tubes at low speed for 1 minute.

4. Gently shake the tubes and examine macroscopically and microscopically for agglutination.

5. At the same time it is advisable to set up a fourth tube in the same manner using the patient's serum and own corpuscles for possible auto-agglutination; this tube should be incubated at room temperature.

**Slide Method.**—1. Arrange 2 clean slides for each test; at one end of slide No. 1 mix a drop of the patient's corpuscle suspension with a drop of the donor's serum and a drop of saline solution. At the other end mix a drop of the patient's corpuscles

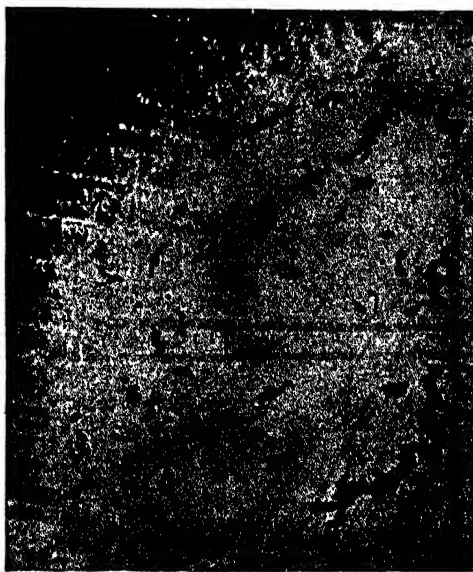


FIG. 299.—A POSITIVE REACTION

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

5. Allow to stand for 15 minutes at room temperature.

6. Examine microscopically with low magnification. The controls should be examined first and should show no agglutination. False clumping and rouleaux formation at the margins of the mixtures should not be mistaken for positive reactions (Figs. 297, 298 and 299).

### METHOD FOR DETECTING SUBGROUPS OF A AND AB

As previously stated, these occur as  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_1B$ ,  $A_2B$  and  $A_3B$ .  $A_3$  and  $A_3B$  are quite rare. Reactions do not usually occur in first transfusions of group A patients with  $A_1$ ,  $A_2$  or  $A_3$  corpuscles. The same is true in the case of first transfusions of group AB patients with the corpuscles of  $A_1B$ ,  $A_2B$  or  $A_3B$  donors. Multiple transfusions of group A and group AB patients with these subgroups, however, may result in the production of sufficient amounts of agglutinins to produce transfusion reactions. The same is true of first or multiple transfusions of women belonging to groups A and AB during pregnancy or the puerperium who may have developed agglutinins for the subgroups through iso-immunization by the fetus.

**Testing Serum.**—Group B serum usually contains not only the common anti-A agglutinin which agglutinates corpuscles A,  $A_1$ ,  $A_2$ ,  $A_1B$  and  $A_2B$  but also anti- $A_1$  agglutinin which agglutinates only  $A_1$  and  $A_1B$  corpuscles. Consequently, when the common anti-A agglutinin is removed by absorption only the anti- $A_1$  agglutinin remains. Therefore if absorbed B serum is used, agglutination shows that the corpuscles belong to subgroups  $A_1$  or  $A_1B$ ; if no agglutination occurs, the corpuscles belong to subgroups  $A_2$  or  $A_2B$ .

Absorbed group B serum may be prepared as follows: (a) If the serum is fresh inactivate by heating in a water bath at  $56^\circ$  C. for 10 minutes; (b) draw blood from an individual belonging to subgroups  $A_2$  and place in citrate solution to prevent coagulation; centrifuge, wash the corpuscles twice with saline solution and discard the supernatant fluid; (c) mix the serum with  $\frac{1}{4}$  its volume of the packed  $A_2$  corpuscles; (d) allow to stand at room temperature for 2 hours with occasional shaking; (e) centrifuge and separate the serum; (f) to each cc. of the serum add 0.2 cc. of 1:1000 aqueous solution of merthiolate as a preservative, label and keep in a refrigerator.

**Test.**—The test is conducted with absorbed group B serum in the same manner as the ordinary grouping tests by the slide or test tube methods described above. With good absorbed B serum, all  $A_1$  corpuscles are strongly agglutinated;  $A_1B$  corpuscles are not as markedly agglutinated. As previously stated,  $A_2$  and  $A_2B$  corpuscles show no trace of agglutination even upon microscopic examination.

### METHOD FOR DETECTING SUBGROUP Rh

As previously stated, the corpuscles of about 85 per cent of white individuals contain the agglutinin Rh discovered by Landsteiner and Wiener (*Proc. Soc. Exper. Biol. and Med.* 43: 223, 1940) about  $\frac{1}{6}$  belonging to subtype  $Rh_2$ . Agglutinins for this agglutinin do not occur normally in sera. But agglutinins may be developed by multiple transfusions with  $Rh+$  corpuscles and by iso-immunization of women during pregnancy by  $Rh+$  fetuses. These acquired agglutinins may produce hemolytic transfusion reactions and play a role in the etiology of erythroblastosis fetalis.

**Testing Sera.**—1. Human testing sera containing anti-Rh agglutinins may be obtained from certain patients who have had hemolytic transfusion reactions and often from mothers of infants with erythroblastosis fetalis. Unless the individual belongs to group AB, it is necessary to remove anti-A and anti-B agglutinins by absorption with group A and group B corpuscles. Otherwise these agglutinins may be removed from the serum by absorption with the saliva of known secretors according to method of Wiener as follows: (a) Pool 2 parts of A saliva and 1 part of B saliva; (b) place in boiling water for 10 minutes, centrifuge and pipet off the supernatant opalescent ("purified") saliva (keeps indefinitely when stored in the refrigerator under sterile conditions); (c) mix 2 parts of serum with 1 part of saliva, allow to stand at room temperature for an hour or two and centrifuge; (d) remove supernatant serum and heat in water bath at 56° C. for 10 to 20 minutes.

2. Testing sera, however, are usually prepared by the immunization of guinea-pigs with the corpuscles of *Macacus rhesus* monkeys as follows: (a) Collect blood in sterile citrate solution to prevent coagulation, wash 2 to 3 times with saline solution and discard the supernatant fluid; (b) prepare a 20 per cent suspension of the packed cells in saline solution and give each of 10 large healthy guinea-pigs an intraperitoneal injection of 5 cc. every 5 days for 5 injections, or 3 injections at weekly intervals of 5, 15 and 15 cc. respectively; (c) bleed the animals 1 week after the last injection and collect the serum of each animal separately; (d) titrate each serum for Rh agglutinin and pool those found satisfactory; (e) inactivate by heating in a water bath at 56° C. for 15 minutes; (f) absorb any anti-A or anti-B agglutinin that may be present by adding to each 2 cc. of serum 0.5 cc. of washed packed group A corpuscles and 0.5 cc. of washed packed group B corpuscles; mix, allow to stand at room temperature for an hour and centrifuge; separate the serum and to each cc. add 0.2 cc. of 1:1000 aqueous solution of merthiolate as a preservative. The group A and B corpuscles should be Rh— since Gallagher and Jones (*Jour. Immunol.* 43: 9, 1943) have shown that it is possible and also advantageous to enhance the selectivity of certain guinea-pig sera by absorption with Rh— human corpuscles which serves to remove antibodies extraneous to the Rh factor.

3. The titration of each guinea-pig serum for Rh agglutinin is conducted as described on page 650 for the titration of group A and group B agglutinins in human serum. The tests must be conducted with the corpuscles of 5 to 10 or more human beings, irrespective of blood groups, in order to include Rh+ and Rh— cells. Those sera which agglutinate at 1:10 or higher are satisfactory as they usually give definite reactions with Rh+ human corpuscles and negative reactions with Rh— corpuscles.

**Test.**—1. Prepare a 2 per cent suspension of *fresh* washed corpuscles in saline solution.

2. In a small test tube (inside diameter 7 mm.) place 2 drops of diluted guinea-pig anti-Rh serum and 1 drop of corpuscle suspension.

3. Mix and place in a water bath at 37° C. for 1 hour.

4. Read the reaction by making a direct inspection of the bottom of the tube, preferably with the aid of a hand lens, noting whether the sediment is compact and smooth or diffuse and rough. Gallagher and Jones have found that the reading is facilitated by holding the tube above the concave side of a microscope mirror laid on the table. A fluorescent desk lamp provides excellent illumination for this purpose. A negative reaction shows a circular deposit with a smooth edge. A positive reac-

tion is characterized by a larger deposit of a wrinkled or granular appearance. Upon gentle shaking a microscopic examination may be made but at times the agglutination is quite weak or even absent in spite of a distinctly positive sediment reading. If agglutination is not visible centrifuge the tubes at 500 to 700 r.p.m. for 1 minute, after which the sediments are again examined for agglutination. Recheck by microscope examinations of the gently resuspended cells in the test tube and on a slide (Diamond). Controls with known positive and negative Rh corpuscles should be included whenever possible.

5. If human testing serum is used, the test is conducted in the same manner except that the tube should be incubated at 37° C. for an hour instead of at room temperature (Levine). As shown by Weiner and Peters, however, some human sera react best in the cold. In general, therefore, the test with human serum should be conducted at 37° C., room temperature and in the refrigerator. When a rapid diagnosis is essential the tube may be centrifuged at low speed for 1 minute after incubation at 37° C. for 10 minutes and the sedimented corpuscles dislodged by gentle shaking with macroscopic and microscopic examinations for agglutination.

6. Taylor and his colleagues (*Brit. Med. Jour.* 572, 1942) have advised setting up the tests with varying dilutions of anti-Rh serum in order to avoid falsely negative reactions due to freeze reactions.

### COLD AGGLUTINATION TEST FOR PRIMARY ATYPICAL PNEUMONIA

As shown by Peterson, Ham and Finland (*Science*, 97: 167, 1943) autohemagglutinins or so-called cold agglutinins, may occur in high titer in the sera of individuals with primary atypical pneumonia. This autohemagglutination does not occur regularly in any other disease except African trypanosomiasis although it has been observed in a few cases of lobar pneumonia by McCombs and McElroy (*Arch. Int. Med.* 59: 107, 1937). The method described by Horstmann and Tatlock (*Jour. A. M. A.* 122: 369, 1943) is as follows:

1. *Fresh* serum should be employed as stored sera may give falsely negative reactions.

2. In a series of 11 small test tubes place 1 cc. of dilutions of serum 1:4, 1:8, 1:16, 1:32, etc., up to 1:4096. Tube No. 12 should carry 1 cc. saline solution as a control.

3. To each tube add 0.1 cc. of a 2 per cent suspension of washed human erythrocytes belonging to *group O*.

4. Mix and place in refrigerator at 0 to 4° C. overnight.

5. Make reading. If positive, allow tubes to stand at room temperature for several hours when a second reading is made to ascertain whether the reaction is reversible at room temperature and therefore a true cold agglutination.

6. Positive reactions are interpreted in graduations from 4 plus (a tight disk of erythrocytes on inverting the tube three times) to 1 plus (a fine granular appearance on similar agitation).

**MEDICOLEGAL APPLICATIONS OF BLOOD GROUPING TESTS**

**Disputed Parentage.**—1. The agglutinogens A, B, M and N are transmitted by heredity.

2. Examinations in relation to disputed parentage are mainly based upon determinations of the major blood groups (O, A, B, AB) of the man, woman and child. Examinations of the subgroups of A and AB corpuscles are not employed.

3. As shown in Table 28, agglutinogens A and B cannot be present in the corpuscles of a child unless present in the corpuscles of either or both parents.

4. A child belonging to group O cannot have a parent belonging to group AB.

5. A child belonging to group AB cannot have a parent belonging to group O.

6. The tests may prove the nonpaternity of an accused man. However, they cannot prove his paternity because some other man of the same blood group may be the father.

7. If the man is group AB and the child group O, or vice versa, nonpaternity is established, regardless of the blood group of the mother. If both man and child are group A, nonpaternity cannot be established.

8. The chances of establishing nonmaternity by the major blood groups alone are slight because of the low incidence of group AB.

9. Maternity is disproven if an alleged mother is group AB and the child group O, or the reverse.

10. Supplementary examinations for the agglutinogens M and N are of additional value and sometimes essential.

11. As shown in Table 29, men belonging to type MN cannot prove nonpaternity. If the child is type M or N and these are absent in one or both parents, nonpaternity is established; also if the man is type M and the child type N, or the man is type N and the child is type M.

12. Maternity is disproven if an alleged mother is type M and the child type N, or the reverse.

13. In cases involving the parentage of infants accidentally interchanged in a hospital, or purposely exchanged by wet nurses, tests for agglutinogens M and N alone solve 40 per cent and tests for agglutinogens A, B, M and N about 70 per cent.

**TABLE 28.—MAJOR BLOOD GROUPS IN RELATION TO DISPUTED PARENTAGE**

Groups of Parents	Groups of Children Possible	Groups of Children Not Possible
O x O	O	A, B, AB
O x A	O, A	B, AB
O x B	O, B	A, AB
O x AB	A, B	O, AB
A x A	O, A	B, AB
A x B	O, A, B, AB	—
A x AB	A, B, AB	O
B x B	O, B	A, AB
B x AB	A, B, AB	O
AB x AB	A, B, AB	O

TABLE 29.—TYPES M AND N IN RELATION TO DISPUTED PARENTAGE

Groups of Parents	Groups of Children Possible	Groups of Children Not Possible
MN x MN	MN, M, N	—
MN x N	MN, N	M
MN x M	MN, M	N
M x N	MN	M, N
M x M	M	MN, N
N x N	N	MN, M

**Blood, Seminal and Other Stains.**—1. Tests for iso-agglutinins and iso-agglutinogens in blood stains possess positive but no negative value in determining the source of human blood.

2. Extracts of dried semen may contain the major blood agglutinogens which may aid in determining the source of a stain.

3. Urine and extracts of dried saliva may contain the major agglutinogens corresponding to those in the erythrocytes of human beings.

### METHODS FOR DETECTING AGGLUTINOGENS M AND N

The erythrocytes of all human beings contain the agglutinogens M, N or MN discovered by Landsteiner and Levine (*Proc. Soc. Exper. Biol. and Med.* 24: 600, 941, 1927). They are unrelated to the agglutinogens A and B. Agglutinins for M, however, occur very rarely in human sera and none have been discovered for N. Furthermore, both agglutinogens are of low antigenic activity so that reactions due to the production of agglutinins for them are but rarely produced by multiple transfusions.

**Testing Sera.**—Consequently, testing sera must be prepared by the immunization of rabbits. Only some animals produce satisfactory sera. Agglutinin for M is produced more effectively than for N. The following method may be employed:

1. Give each of a series of healthy adult rabbits a daily intravenous injection of 1 cc. of a 10 per cent suspension of washed M corpuscles from a group O donor for 3 or 4 weeks. Inject a second series in the same manner with N corpuscles from a group O donor.

2. Bleed the rabbits about 1 week after the last injection and separate the sera. Inactivate the sera in a water bath by heating at 56° C. for 15 to 20 minutes.

3. Dilute each anti-M serum 1:20 with saline solution and add a half volume of packed, washed N corpuscles. Mix, incubate at 37° C. for 1 hour and centrifuge. Pipet off the supernatant serum and test for M and N agglutinins as follows: (a) In a series of 7 small test tubes place 0.2 cc. of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 dilutions of serum; (b) add 0.2 cc. of a 2 per cent suspension of M corpuscles to each (final dilutions of serum are now 1:4 to 1:256); (c) set up a second series of test tubes in the same manner using N corpuscles; (d) mix all tubes and place in water bath at 37° C. for an hour when the readings are made. A satisfactory serum is one showing agglutination of M corpuscles in final dilution of 1:64 or higher with no agglutination of N corpuscles. Any serum showing agglutination of N corpuscles must be re-absorbed with N corpuscles as described and re-tested.

4. Dilute each anti-N serum 1:20 with saline solution and absorb with M corpuscles as described. Test each serum for agglutinins for M and N corpuscles in the same manner. A satisfactory serum should agglutinate N corpuscles in a final dilution of at least 1:16 and show no agglutination of M corpuscles. If agglutinins for M corpuscles are present, re-absorb with M corpuscles and re-test the serum for agglutinin.

5. Pool the satisfactory anti-M sera and to each cc. add 0.2 cc. of 1:1000 aqueous solution of merthiolate as a preservative. Pool the satisfactory anti-N sera and preserve in the same manner.

**Tests.**—1. Prepare a 2 per cent suspension of the fresh washed corpuscles of the unknown blood in saline solution. It is advisable to also prepare similar suspensions of known M and N corpuscles as controls.

2. Arrange 4 small test tubes (inside diameter 7 mm.). In Nos. 1 and 2 place 1 drop of the unknown corpuscle suspension; add 1 drop of proper dilution of anti-M serum to No. 1 and 1 drop of proper dilution of anti-N serum to No. 2. In No. 3 place 1 drop of M corpuscles and 1 drop of anti-M serum (control). In No. 4 place 1 drop of N corpuscles and 1 drop of anti-N serum (control).

3. Mix the tubes and incubate for 1 hour in a water bath at 37° C. or 2 hours at room temperature.

4. Examine the tubes for agglutination both macroscopically and microscopically. Agglutination in tubes 1 and 3 only indicates that the corpuscles of the unknown blood belong to type M; agglutination in tubes 2 and 4 only indicates that the corpuscles of the unknown blood belong to type N; agglutination in tubes 1 and 2 indicates that the corpuscles of the unknown blood belong to type MN.

### DONATH-LANDSTEINER TEST FOR PAROXYSMAL HEMOGLOBINURIA

**Procedure.**—1. Obtain blood by venous puncture. Add a small portion to 10 volumes of 0.85 per cent saline solution. The remainder is placed in a dry tube and allowed to clot. The syringe, tubes and saline solution should be warm and the blood kept warm during the preparation of the serum and 5 per cent cell suspension.

2. Wash the cells three times in warm 0.85 per cent saline solution.

3. Centrifuge the clotted blood and remove the serum as soon as possible.

4. Obtain blood in the same manner from a normal individual of the same blood group as the patient (control) and prepare serum and cell suspension.

5. Prepare a 1:10 dilution of fresh guinea pig serum (complement).

Tube	Patient's serum	Patient's R.B.C. 5%	Control Serum	Control R.B.C. 5%	Comp. 1:10	Saline 0.85%	
1	0.25 cc.	0.1 cc.	.....	.....	0.1 cc.	0.05 cc.	Immerse in ice water — 10 min. Water-bath at 37° C. —30 min.
2	.....	.....	0.25 cc.	0.1 cc.	0.1 cc.	0.05 cc.	
3	0.25 cc.	.....	.....	0.1 cc.	0.1 cc.	0.05 cc.	
4	.....	0.1 cc.	0.25 cc.	.....	0.1 cc.	0.05 cc.	
5	.....	0.1 cc.	.....	.....	0.1 cc.	0.3 cc.	
6	.....	.....	.....	0.1 cc.	0.1 cc.	0.3 cc.	

6. Set up control test except that chilling in ice water is omitted.

7. A positive reaction is indicated by hemolysis in tubes 1 and 3 of the first set chilled in ice water for 10 minutes followed by water bath at 37° C. for 30 minutes. There should be no hemolysis in the control or unchilled set.

# METHODS FOR CONDUCTING COMPLEMENT FIXATION TESTS FOR SYPHILIS AND OTHER DISEASES

## GENERAL TECHNIQ

The glassware and apparatus and methods for the preparation of saline solution, complement, hemolysin and sheep corpuscles are essentially the same for the different complement fixation tests herein described.

**Glassware and Apparatus.**—1. Accurate *pipets* are essential. These should include 1 cc. pipets graduated in 0.01 cc. to the tip, 5 cc. pipets graduated in 0.1 cc. and 10 cc. pipets graduated in 0.1 cc. Electrically driven automatic pipets capable of delivering 0.1 to 1.0 cc. volumes with an accuracy within at least 0.02 cc. are convenient for pipetting saline solution, antigen, complement, corpuscle suspension and hemolysin.

2. *Test tubes* (85 by 13 mm. inside diameter) with rounded bottoms and no lips may be used. Galvanized wire racks carrying 12 rows of 6 tubes each are convenient.

3. Any easily regulated water bath is satisfactory; electric baths with automatic thermo-regulators are recommended.

4. Any refrigerator capable of maintaining a constant temperature of 0 to 8° C. is satisfactory.

**Method of Cleaning Glassware.**—1. All glassware should be chemically clean and preferably sterile. To clean tubes and flasks, empty and rinse in running tap water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in the hot air oven at about 160° C.

2. Pipets should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean pipets, rinse thoroughly in running tap water, place in a metal box or wire basket and sterilize in the oven.

3. Flasks should be plugged with cotton and sterilized in the oven for 30 minutes at 160° C.

4. If glassware becomes cloudy, immerse in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid, and 25 parts water) for 24 hours. Rinse thoroughly in running tap water and proceed with the washing as described.

**Preparation of Saline Solution.**—1. Thoroughly dry C.P. sodium chloride in a hot air oven and dissolve 8.5 gms. in 1000 cc. of freshly distilled water (0.85 per cent solution). It is convenient to weigh and dispense this amount of sodium chloride in tightly stoppered test tubes sufficient for a month and thereby avoiding the necessity of drying and weighing each time a fresh solution is required. Otherwise a stock solution of 17 per cent sodium chloride in distilled water may be prepared and 50 cc. diluted with 950 cc. of distilled water as saline solution (0.85 per cent) is required.

2. It is advisable but not always essential to add 1.0 cc. of a 10 per cent solution of magnesium sulfate in distilled water to each 1000 cc. of solution as recommended by Kellogg.

3. Filter through paper into a flask fitted with a gauze-covered cotton stopper. Sterilize by heating in an Arnold sterilizer for 1 hour before use (not essential if immediately used).

4. A satisfactory saline solution should not be hemolytic when 1 or 3 drops of



washed sheep corpuscles are added to 5 cc. in a test tube followed by water bath incubation at 37° C. for an hour. Neither should it be anticomplementary in complement and hemolysin titrations.

**Preparation of Sheep Corpuscles.**—1. The method of choice is to collect blood from an external jugular vein as aseptically as conditions permit as described on page 32. Otherwise *fresh* sheep blood should be collected at an abattoir.

2. If preservation is not desired, the blood must be immediately defibrinated after collection by thorough shaking with glass beads.

3. Collection in an anticoagulant and preservative solution is recommended. For this purpose 1 part of the following solution (Boerner and Lukens) for each 9 parts of blood is satisfactory:

Sodium citrate .....	8.0 gm.
Dextrose .....	20.0 gm.
1:1000 aqueous solution of merthiolate .....	100.0 cc.

The following method (Kolmer) is likewise satisfactory: In a clean (but not necessarily sterile) quart-sized mason jar, place 30 cc. of a 10 per cent solution of sodium citrate in saline solution and 2 cc. of formalin. At the abattoir have the jar almost filled with *fresh* blood, screw on the top and mix thoroughly.

Blood collected by either method generally remains serviceable for 1 to 3 weeks if stored at 0 to 5° C. Boerner and Lukens advise keeping blood in a refrigerator for 48 hours before using.

4. For use, filter a small quantity of blood through cotton into a *graduated* centrifuge tube. Allow twice as much blood as the amount of cells required. Add 2 or 3 volumes of saline solution. Centrifuge at a moderate velocity until it is ascertained that all the corpuscles are thrown down. Remove the supernatant fluid with a capillary pipet or by suction (Fig. 300). Add 3 or 4 volumes of saline solution; mix by inverting and centrifuge again for the same length of time. Repeat the process for a third time but centrifuge twice as long as in the first washing in order to pack the cells evenly and firmly. Cells should be washed until the supernatant fluid is almost colorless. Three washings are usually sufficient. (If more than 4 washings are necessary, the cells are too fragile for use.) Carefully remove the supernatant fluid.

**Preparation of Antisheep Hemolysin.**—Give a rabbit 5 or 6 intravenous injections of 5 cc. of a 10 per cent suspension of washed sheep corpuscles every 5 days. Bleed the rabbit 7 to 9 days after the last injection if a preliminary titration is satisfactory. Separate the serum (it need not be inactivated) and preserve with an equal part of best grade neutral glycerin. Keep in a refrigerator.

**Preparation of Complement.**—1. As shown by Giordano and Carlson (*Am. Jour. Clin. Path.* 9: 130, 1939) it is advisable to pretest the sera of guinea-pigs individually for nonspecific reactions before use as complement in the conduct of complement fixation tests for syphilis. These preliminary tests should be conducted with the same antigen as employed in the syphilis tests because, as shown by Harris (*Jour. Lab. and Clin. Med.* 27: 3, 1941), nonspecific reactions may occur with one antigen and not with another. Mixtures of the sera of 100 or more guinea-pigs, however, are rarely unsatisfactory. When such are used, the complement may be pretested after pooling. If found to yield nonspecific reactions with one antigen, it should be tested with others as it is extremely unlikely that any pools will be found that are not satisfactory

with at least one of them. The technics employed for pretesting complement for the Boerner-Lukens and Kolmer complement fixation tests are described below.

3. The pooled sera of at least 3 to 5 healthy guinea-pigs should be used. Select large, well-nourished animals that have not been fed for at least 12 hours; avoid pregnant animals.

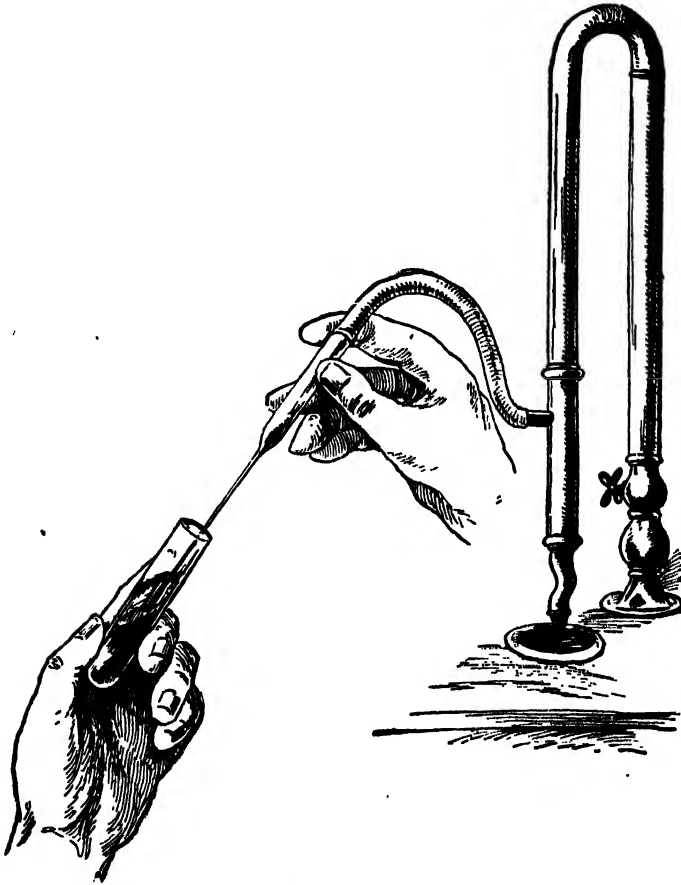


FIG 300.—SUCTION PUMP

(From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

4. An excellent and economical procedure is to maintain a colony of guinea-pigs and to remove 4 to 5 cc. of blood from the hearts of a sufficient number in centrifuge tubes to yield the required amount of complement serum. The animals may be bled in rotation every 4 to 6 weeks. The technic is described on page 31. Otherwise each animal may be exsanguinated as follows: Anesthetize the pig lightly with ether or stun the animal with 1 or 2 sharp blows on the head; sever the large blood vessels on both sides of the neck, being careful not to cut the esophagus or trachea; collect the blood in centrifuge tubes by means of a large funnel. With either method Kolmer advises placing the blood in an incubator at 37° C. for an hour before the clots are broken up and centrifuged for the collection of serum. Otherwise they may be kept at

room temperature for an hour or two and then placed in a refrigerator overnight when the sera are separated.

5. Pooled complement sera may be preserved by various methods. (a) The most satisfactory is by evaporation of 5 cc. amounts in separate ampules in the frozen state in vacuo by the lyophile or cryochem methods described on pages 618 to 619. It usually retains both hemolytic activity and fixability for 8 to 12 months if kept at a low temperature in a refrigerator. By adding 5 cc. of distilled water the material goes into immediate solution upon gentle shaking and is ready for use in the same manner as fresh serum.

(b) Otherwise Rhamy's sodium acetate method as modified by Sonnenschein (*Ztsch. f. Immunitätsf.* 67: 512, 1930) may be employed by adding to the complement serum an equal part of a solution prepared by dissolving 12 gms. sodium acetate and 4 gms. boric acid in 100 cc. distilled water. For use 2 cc. diluted with 8 cc. of saline solution gives a 1:10 dilution of complement or 1 cc. diluted with 14 cc. of saline solution a 1:30 dilution. (c) Preservation with sodium chloride may also be employed by adding 1.0 gm. per 10 cc. of serum and mixing thoroughly. For use 1 cc. diluted with 9 cc. of distilled water gives a 1:10 dilution in isotonic saline solution from which a 1:30 dilution may be prepared. In either case the preserved complement should be kept in a nearly frozen state in the freezing compartment of a mechanical refrigerator. As the complement loses first in fixability by syphilis antigen and reagin, it may not be satisfactory for longer than 3 or 4 weeks.

**Preparation of Sera.**—1. All specimens are lined up and properly labelled.

2. The sera are removed from the clots with capillary pipets to test tubes properly labelled. Great care is required to prevent errors in labelling and confusion of sera. Each serum should be free of corpuscles, otherwise it is necessary to break up the clots with wooden applicators (one for each serum) and centrifuging for clear serum. Slight tinging with hemoglobin does no harm. Sera containing large amounts of hemoglobin are likely to be anticomplementary and unsatisfactory for both complement fixation and flocculation tests.

3. It is not necessary to remove the natural antisheep hemolysins by absorption with *thoroughly washed sheep corpuscles* although this tends to increase the sensitiveness of complement fixation reactions and especially in the case of sera containing small amounts of syphilis antibody. Kolmer recommends the routine removal of natural hemolysins when conditions permit in order to secure reactions of maximum sensitiveness, but when large numbers of sera require testing it may be omitted. The method for removal of natural antisheep hemolysins from sera is as follows:

(a) To each is added a drop of washed sheep corpuscle sediment for approximately each 2 cc. of blood and serum as gaged by inspection.

(b) Each specimen is then thoroughly mixed with a wooden applicator (1 for each serum).

(c) All are then placed in a refrigerator for 15 minutes to enable the sheep corpuscles to absorb the hemolysins with none or but a minimum and harmless amount of hemolysis.

(d) All specimens are now centrifuged and the sera separated into test tubes properly labelled.

(e) The sera are then heated in a water bath, the temperature and duration varying according to the method employed. If serum has been heated a day or two before

the actual test, it should be reheated for 5 minutes at 56° C. before proceeding with the test.

**Preparation of Spinal Fluids.**—*These are usually tested without any preliminary preparation* as they do not contain enough natural antishoop hemolysin to require removal or enough complement to require inactivation by heating at 55° C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged. Otherwise no preparation is required as spinal fluids are tested as delivered without preliminary heating unless they are more than 3 days old, in which case they may be heated at 55° C. for 15 minutes to remove thermolabile anti-complementary substances. As a general rule, however, spinal fluids are not anti-complementary unless heavily contaminated with bacteria (cloudy).

### BOERNER-LUKENS COMPLEMENT FIXATION TEST FOR SYPHILIS

**Preparation of Antigen.**—1. Place 10 gms. of powdered beef heart (Difco) in a flask of 1000 or 2000 cc. capacity.

2. Add 225 cc. of absolute alcohol and 75 cc. of ether (U.S.P.) and allow to stand at room temperature for ½ hour, shaking thoroughly every 5 minutes or shake continuously for 15 minutes.

3. Filter through filter paper and discard the residue.

4. Place the filtrate in a large flask or beaker and evaporate to 50 cc. by boiling in a water bath or on a hot plate. If the concentrated extract is less than 50 cc., add sufficient absolute alcohol to this amount.

5. Place the concentrated filtrate in the refrigerator at 6° to 8° C. overnight. A heavy precipitate will form.

6. Filter through paper. This filtration should be done in the refrigerator so that the filtration will be completed while the solution is still cold. It is advisable to place the funnel, filter paper, and flask in the refrigerator about ½ hour before filtering.

7. Add 4 mg. of cholesterol to each cc. of filtrate and warm in water bath until dissolved. This is the finished antigen.

8. Antigens prepared by the above method are slightly more anti-complementary than the alcohol used in their preparation. It is therefore unnecessary to titrate for anti-complementary properties. *The dilution of antigen to be employed in the test is 1:1000.* This dose was determined after a study of the optimum dose of over 100 antigens prepared according to the above method. Each lot of antigen should be tested for its antigenic properties by running a series of tests with positive serum diluted with negative and compared with other satisfactory antigens run at the same time.

**Hemolytic System and Its Adjustment.**—1. Prepare a 1.5 per cent suspension of packed sheep erythrocytes in saline solution.

2. Prepare a 1:20 stock solution of antishoop hemolysin by mixing 2 cc. of glycerinated hemolysin (50 per cent), 9 cc. of saline solution and 9 cc. of neutral glycerine.

3. Dilute 2 cc. of complement preserved by the Rhamy method as modified by Sonnenschein, with 28 cc. of saline solution (1:30) or 1 cc. of fresh undiluted complement with 29 cc. of saline solution (1:30). Complement preserved by the lyophile or cryochem processes should be restored by adding distilled water or preservative in amount equal to the original amount of guinea-pig serum placed in the container.

This restored complement is then diluted 1:30 by placing 1 cc. in 29 cc. of saline solution.

4. Prepare a 1:75 dilution of complement by diluting 4 cc. of 1:30 complement with 6 cc. of saline solution.
5. Arrange 8 tubes in a series and mark them 1 to 8 inclusive.
6. Prepare a 1:100 dilution of antish sheep hemolysin in complement by adding 0.1 cc. of the 1:20 stock solution of hemolysin to 0.4 cc. of 1:75 complement dilution. Make further dilutions of hemolysin as shown in Table 30.
7. Discard 0.5 cc. from tubes 3, 7, and 8.
8. To all tubes add 0.5 cc. of 0.75 per cent sheep cell suspension prepared by using an equal volume of 1.5 per cent cell suspension prepared for use in the test, and an equal volume of saline.
9. Mix by shaking rack and place in water bath at 37° C. for 30 minutes.
10. The titer is the highest dilution of hemolysin showing complete hemolysis. For sensitizing the cells to be used in the test proper, 4 times this amount is used. This dilution is prepared from the 1:20 stock solution of hemolysin. A complement not producing complete hemolysis in at least 1:5000 dilution of hemolysin is considered unsatisfactory.

**TABLE 30.—ADJUSTING THE HEMOLYTIC SYSTEM**

Tube	Complement 1:75	Hemolysin		Hemolysin Dilution
1	0.9 cc.	0.1 cc. of 1:100	≈	1:1000
2	0.8 cc.	0.2 cc. from tube 1	=	1:5000
3	1.3 cc.	0.2 cc. from tube 1	=	1:7500
4	0.5 cc.	0.5 cc. from tube 2	=	1:10,000
5	0.5 cc.	0.5 cc. from tube 3	=	1:15,000
6	0.5 cc.	0.5 cc. from tube 4	=	1:20,000
7	0.5 cc.	0.5 cc. from tube 5	=	1:30,000
8	0.5 cc.	0.5 cc. from tube 6	=	1:40,000

11. Example: Titer—1:20,000.

Four times this amount = 1:5000

Diluting from 1:20 stock = 1 cc. of 1:20 hemolysin plus 249 cc. saline solution. Smaller amounts can be prepared, depending on the number of tests to be done. To the hemolysin thus prepared an equal volume of 1.5 per cent sheep cell suspension is added. This mixture must stand at least 15 minutes before using in the test to insure sensitization of the cells.

**Pretesting of Complement.**—1. For each guinea-pig serum or lot of pooled sera to be tested arrange 2 tubes.

2. To tube 1 add 2.9 cc. of saline solution if the complement is diluted with preservative; to the second tube add 0.5 cc. of saline solution. If the complement is not diluted with preservative, 2.95 cc. of saline solution should be added to tube 1.

3. To tube 1 add 0.1 cc. of preserved serum. This gives a 1:60 dilution of serum. If complement is not diluted with preservative, 0.05 cc. should be added to tube 1.

4. Mix tube 1, discard 2.0 cc. and transfer 0.5 cc. to tube 2; mix tube 2, and discard 0.5 cc. This gives a 1:60 and a 1:120 dilution of serum.

5. Prepare a 1:200 dilution of antigen by first making a 1:10 by adding 0.1 cc. of antigen drop by drop to 0.9 cc. of saline; then dilute to 20 cc. with saline.

6. To each of the 2 tubes of diluted complement add 0.1 cc. of the 1:200 antigen dilution.

7. Incubate all tubes at 6° to 8° C. for 18 hours.

8. Place in water bath at 37° C. for 10 minutes.

9. To all tubes add 0.5 cc. of 0.75 per cent sensitized sheep cells.

10. Mix well by shaking and place in water bath at 37° C. for ½ hour.

11. Sera giving complete hemolysis in the 1:60 dilution are considered satisfactory. Pool all the satisfactory sera and store in the frozen state in amounts equal to each day's work. Sera giving slight inhibition in the 1:60 may be tested with another antigen and if satisfactory may be used with that antigen, or it may be mixed with 5 or more sera which give complete hemolysis in the 1:120 dose.

**Preparation of Antigen-Complement Mixture (A-C Mixture).—**1. Estimate the amount of A-C mixture required, allowing 0.5 cc. for each dose.

2. Prepare a 1:30 dilution of complement (sufficient amount for tests).

3. Prepare a 1:10 dilution of antigen by adding 0.1 cc. of antigen drop by drop to 0.9 cc. of 1:30 complement.

4. Dilute 1:10 antigen dilution further to a 1:1000 dilution in 1:30 complement. A simple method for calculating amount of 1:10 antigen to be added to the complement to make the A-C mixture is as follows: Divide the amount of A-C mixture to be used by 100. This gives the amount of 1:10 antigen to add to sufficient complement to make this amount.

**Complement Fixation Test (Qualitative).—**1. For each serum to be tested, 2 tubes are used (Kolmer or Kahn). To each tube add 0.1 cc. of serum (Table 31). Place in water bath at 58° C. for 10 minutes, or the serum may be inactivated at 56° C. for 30 minutes before placing in tubes. The latter is often more desirable when other tests are to be performed on the same sera.

2. Include 3 reagent controls, antigen, hemolytic and corpuscle.

3. To tube 1, add 0.5 cc. of A-C mixture.

4. To tube 2, add 0.5 cc. of 1:30 complement (serum control).

5. Mix by shaking rack. Place in icebox at 6° to 8° C. for 15 to 18 hours.

6. Place in water bath at 37° C. for 10 minutes.

7. To both tubes add 0.5 cc. of 0.75 per cent sensitized sheep cell suspension (cells should be sensitized 15 minutes before using).

8. Mix by shaking rack and place in water bath at 37° C. for ½ hour.

9. Read the reactions as follows:

<i>Hemolysis</i>	<i>Report</i>
100 per cent inhibition	plus 4 = Positive
75 per cent inhibition	plus 3 = Weakly positive
50 per cent inhibition	plus 2 = Weakly positive
25 per cent inhibition	plus 1 = Doubtful
12.5 per cent inhibition	plus-minus = Doubtful
Less than 12.5 per cent inhibition	= Negative

10. Anticomplementary sera should be repeated with 0.05 cc. of serum. If serum is still anticomplementary on retesting with the smaller amount of serum, a repeat specimen should be requested.

TABLE 31.—SIMPLIFIED COMPLEMENT FIXATION TEST (QUALITATIVE)

Tube	Patient's Serum cc.	A-C Mixture cc.	Comp. 1:30 cc.	Incubate 15-18 hours at 6° - 8° C. followed by 10 minutes at 37° C.	Sensitized Cells 0.75% cc.	Incubate in water bath at 37° C. for 1 1/2 hour; make readings.
1	0.1	0.5	—		0.5	
2	0.1	—	0.5		0.5	
<i>Controls</i>						
Antigen	—	0.5	—		0.5	
Complement	—	—	0.5		0.5	
Cells	0.5	—	—		0.5	
	Saline					

**Complement Fixation Test (Quantitative).**—1. For each serum to be tested, 3 or more tubes are used.

2. To all tubes except the last add 0.2 cc. of saline.

3. Add 0.2 cc. of inactivated serum to tube 1 and 0.1 cc. to the last tube (serum control).

4. Mix tube 1 and transfer 0.2 cc. to tube 2; mix tube 2 and transfer 0.2 cc. to tube 3; and continue transferring until the next to the last tube is reached. Then discard 0.2 cc.

5. Continue in same manner as described under qualitative test, adding 0.5 cc. of A-C mixture to all tubes except the last. To the last tube, add 0.5 cc. of 1:30 complement.

**Spinal Fluid Complement Fixation Test (Qualitative).**—1. For each fluid to be tested use 2 tubes.

2. Place 0.25 cc. fluid in each tube.

3. Heat at 58° C. for 10 minutes, or at 56° C. for 30 minutes.

4. Continue as in qualitative test for sera.

5. If the complement used is not pretested egg albumin should be added to each tube (0.1 cc. of a 50 per cent solution of egg white in saline solution as recommended by Boerner and Lukens, *Am. Jour. Clin. Path.* 11: 71, 1941; *Am. Jour. Med. Tech.* 8: 114, 1942).

**Spinal Fluid Complement Fixation Test (Quantitative).**—1. The test is the same as described for blood serum, except the spinal fluid is diluted as follows:

To all tubes except the first and last, add 0.25 cc. of saline; then add 0.25 cc. of spinal fluid to the first, second, and last; mix and transfer 0.25 cc. from tube 2 to tube 3, continuing transfers until next to the last tube, then discard 0.25 cc. from this tube.

2. Continue as in qualitative test for sera.

**EAGLE MODIFICATION OF THE WASSERMANN TEST**

**Preparation of Antigen.**—If a uniform preparation of dried powdered beef heart is not available, the technician may prepare his own. The heart tissue must be quite fresh, and the fat, pericardium, and the blood vessels should be removed. The muscle is ground in an ordinary meat grinder, and then dried by the addition of 150 cc. of acetone to each 100 grams of tissue. After about an hour at room temperature, with frequent shaking, the acetone is removed by filtration, the tissue is shaken with a second portion of acetone (150 cc.) for a few minutes, and the mixture again filtered. The acetone filtrates are discarded. The tissue is spread in a thin layer on a clean surface and thoroughly dried in a 37° C. incubator for 24 hours, when it is turned and dried again for 24 hours. The dry sheet is now pulverized as finely as possible in a mortar, or preferably, in a pulverizing machine.

Fifty grams of the dried powdered beef heart, preferably a pooled preparation such as those available commercially (*e.g.*, Difco), are extracted with 250 cc. of pure anesthesia ether (5 cc. per gram powder) for 15 minutes at 30° to 37° C., with frequent shaking. The mixture is filtered with suction, and the extraction is repeated with fresh ether for a total of 4 extractions. All the ether extracts are discarded. After the fourth filtration, the beef heart powder is washed on the filter with 100 cc. of fresh ether, dried, and then extracted for 5 days with 250 cc. of absolute alcohol (5 cc. per gram powder). The alcohol extract is filtered and the moist powder is washed on the filter with small portions of fresh alcohol until the volume of the combined alcoholic filtrate and washings is equal to 250 cc. (5 cc. per gram powder). The clear, straw-yellow extract is the basic antigen, and contains approximately 1.2 to 1.5 per cent solids. It is now fortified with cholesterol which is added to a concentration of 0.6 per cent (6 mg. per cc. extract). The cholesterol is dissolved by boiling. This completed antigen, tightly stoppered, retains its reactivity almost indefinitely (more than 8 years) at room temperature. For use in the test, it is diluted fresh each day by slowly pouring from 80 to 200 volumes of 0.85 per cent salt solution into 1 volume of the antigen. The exact quantity of salt solution to be used is determined once for each lot of antigen by the technic to be described. One cubic centimeter of antigen suffices for approximately 100 tests.

It is to be noted that the same basic extract, fortified with 0.6 per cent cholesterol and 0.6 per cent corn germ sterol, is used in the Eagle flocculation test.

**Hemolytic System and Its Adjustment.**—1. Prepare a stock 3 per cent suspension of washed sheep cells by diluting 1 part of packed cells with 32 parts of saline solution.

2. The complement is used in a 1:10 dilution.

3. The minimal hemolytic quantity (unit) of amboceptor must be determined daily for the particular cell suspension to be used that day, by the technic of Table 32. This hemolytic unit of amboceptor should be a 1:2400 dilution or higher; and an amboceptor should be discarded if it is so inactive that the hemolytic unit represents, *e.g.*, a 1:1000 dilution.

The unit of amboceptor in the example cited is a 1:3000 to 1:4000 dilution, or, interpolating, approximately a 1:3500 dilution. An amboceptor dilution is now prepared containing  $2\frac{1}{2}$  units (in the example cited, a 1:1400 dilution). This amboceptor



dilution is poured into an equal volume of the 3 per cent cell suspension to form the 1½ per cent suspension of sensitized cells which is actually used in the test.

TABLE 32.—TECHNIC OF AMBOCEPTOR TITRATION

	Amboceptor titration *						
	1:1000	1:1500	1:2000	1:3000	1:4000	1:6000	1:8000
Amboceptor dilution, cc. ....	0.4	0.4	0.4	0.4	0.4	0.4	0.4
3 per cent cell suspension, cc. ..	.4	.4	.4	0.4	.4	.4	.4
Salt solution, cc. ....	.8	.8	.8	.8	.8	.8	.8
Complement, 1:10, cc. ....	.4	.4	.4	.4	.4	.4	.4
Example of reading of hemolysis after ½ hour at 37° C. ....	Complete	Complete	Complete	Complete	Partial	Partial	None

\* The following is a simple method of setting up these amboceptor dilutions:

1:1000 amboceptor, cc. ....	0.4	0.27	0.2	0.13	0.10	0.067	0.05
Salt solution, cc. ....	0.0	0.13	0.2	0.27	0.30	0.34	0.35
Final dilution of amboceptor ....	1:1000	1:1500	1:2000	1:3000	1:4000	1:6000	1:8000

4. The sensitization of the cells should be completed within an hour. One set of complement controls previously placed in the refrigerator along with the tests, and containing 0.4, 0.2, 0.13, and 0.1 cc. of complement in a total value of 1.2 cc., is now removed from the refrigerator, and 0.8 cc. of the sensitized cell suspension is added to each tube. The degree of hemolysis is read after 30 minutes at 37° C. If the amboceptor titration was correct, the 0.4 cc. of 1:10 complement used in the test represents 2 to 2½ times the amount necessary to cause complete hemolysis in 30 minutes. Accordingly, the first 2 tubes of the complement titration should be completely hemolyzed; tube 3 should be partially hemolyzed, and tube 4 should show little or no lysis. Any error in the amboceptor titration becomes immediately apparent. If only the first tube is completely hemolyzed, the cells have been inadequately sensitized and more amboceptor should be added. If 3 tubes show complete lysis, an excess of amboceptor has been used. The technician must reconcile himself to a sudden decrease in the sensitivity of the test, or a second portion of blood must be washed and sensitized with the correct amount of amboceptor, somewhat less than that used in the first lot.

**Titration of Antigen.**—1. Each lot of antigen should be tested once for its anti-complementary and hemolytic activity to ensure that these undesirable properties are not so pronounced as to interfere with its use in the test. The technic of these titrations is given in Tables 33 and 34. As shown in the tables the first tube carries 0.4 cc. of undiluted antigen while the remaining six tubes carry 1:2, 1:3, 1:4, 1:6, 1:8 and 1:12 dilutions prepared with saline solution. In the hemolytic titration the results are read after incubation in a water bath at 37° C. for 30 minutes. The same dilutions are used in the anticomplementary titration with a primary incubation of 4 hours at 0 to 5° C. followed by 30 minutes at 37° C. when sensitized corpuscles are added to all tubes followed by incubation at 37° C. for 30 minutes. Antigens prepared by the method just described are usually not anticomplementary in more than a 1:6 dilution and are not significantly more hemolytic than pure alcohol. The use of a 1:120 dilution in the test proper (*vide infra*) thus provides a wide margin of safety.

TABLE 33.—HEMOLYTIC TITRATION OF ANTIGEN

	Antigen dilution *						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Antigen dilution, cc. ....	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, cc. ....	.8	.8	.8	.8	.8	.8	.8
Cell suspension, cc.† .....	.8	.8	.8	.8	.8	.8	.8
<i>Hemolysis is Read After ½ Hour at 37° C.</i>							
Example of reading of hemolysis.	Complete	None	None	None	None	None	None
Conclusion: The antigen is not significantly hemolytic.							

\* These dilutions are readily prepared by the following procedure:

Whole antigen, cc. . . . .	0.4	0.2	0.13	0.1	0.07	0.05	0.035
Salt solution, cc. ....	1	.2	.27	.3	.33	.35	.37
Final dilution of antigen	1	1:2	1:3	1:4	1:6	1:8	1:12

† Can be sensitized or unsensitized, but should be 1.5 per cent by volume.

TABLE 34.—ANTICOMPLEMENTARY TITRATION OF ANTIGEN

	Antigen dilution *						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Antigen dilution, cc. ....	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, cc. ....	.4	.4	.4	.4	.4	.4	.4
Complement, 1:10, cc. ....	.4	.4	.4	.4	.4	.4	.4
After 4 hours of 0° to 5° C., followed by ½ hour at 37° C., add 0.8 cc. of sensitized cells to all the tubes							
Example of reading of hemolysis after ½ hour at 37° C. ....	Complete	0	0	Partial	Complete	Complete	Complete
Conclusion: Antigen is anticomplementary up to 1:4 dilution.							

\* These dilutions are readily prepared by the following procedure:

Whole antigen, cc. . . . .	0.4	0.2	0.13	0.1	0.07	0.05	0.035
Salt solution, cc. ....	0	.2	.27	.3	.33	.35	.37
Final dilution of antigen . . .	1	1:2	1:3	1:4	1:6	1:8	1:12

2. As in the case of the anticomplementary titration, the optimum dilution of the antigen need be determined only once with each lot of antigen. The importance of this titration is not generally realized. A typical titration illustrating the method is given in Table 35. In this example, the most sensitive, and thus the optimum dilution, is approximately 1:140. If the powdered beef heart used for the antigen is a pooled,

uniform product and if the method of preparation is adhered to rigidly, it is generally found that the optimum antigen dilution is also quite uniform, not deviating significantly from the 1:120 to 1:160 value of the following titration.

**TABLE 35.—DETERMINATION OF THE OPTIMUM ANTIGEN DILUTION**

Antigen dilution *	Strongly positive serum	Result of Wassermann test on the same serum diluted with salt solution						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
1:40 .....	+	+	+	±	0	0	0	0
1:80 .....	+	+	+	+	±	0	0	0
1:100 .....	+	+	+	+	+	0	0	0
1:120 .....	+	+	+	+	+	±	0	0
1:160 .....	+	+	+	+	+	±	0	0
1:200 .....	+	+	+	+	+	0	0	0

+ = positive (no hemolysis).

± = doubtful (partial hemolysis).

0 = negative (complete hemolysis).

\* The antigen dilutions to be used in the above-mentioned titration can be prepared as follows:

1:40 antigen dilution, cc..	8.0	4.0	3.2	2.7	2.0	1.6
Salt solution, cc. ....	0	4.0	4.8	5.3	6.0	6.4
Final dilution of antigen .	1:40	1:80	1:100	1:120	1:160	1:200

Similarly, the serum dilutions can be readily prepared as follows:

Serum, cc. ....	4.0	2.0	1.0	0.5	0.25	0.125	0.062
Diluent, cc. ....	0	2.0	3.0	3.5	3.75	3.9	4.0
Final dilution of serum .		1:2	1:4	1:8	1:16	1:32	1:64

In preparing the antigen for daily use in the test, the correct volume of salt solution is slowly poured into 1 volume of antigen. The antigen dilution should be opalescent and homogeneous, containing no visible granules.

**Routine Qualitative Serum Test.**—Three tubes are advisable although only 2 are essential. Tube 1 is the serum control, containing serum and complement, but salt solution instead of antigen. Sera are first heated in a water bath at 56° C. for 15 to 20 minutes.

	Serum control	Test proper	
Whole serum, cc. ....	0.2	0.2	0.1
Complement, 1:10, cc. ....	.4	.4	.4
Antigen dilution, cc. ....	0	.4	.4
0.85 per cent NaCl, cc. ....	.6	*.2	*.3

\* May be omitted.

Tubes 2 and 3 are the test proper, each containing 0.4 cc. complement, 0.4 antigen, and different quantities of serum (0.2 and 0.1 cc.). The addition of salt solution to tubes 2 and 3 is not essential but serves to bring the total volume in all 3 tubes up to 1.2 cc., corresponding to 0.4 cc. of each of the 3 reagents.

One antigen control suffices for the entire series of tests, and 2 complement controls are also set up, as here indicated.

	Antigen control	Complement controls (in duplicate)				
Antigen dilution, cc. ....	0.4	0.4	0.4	0.4	0.4	0.4
Complement, 1:10, cc. ....	.4	.4	.2	.13	.1	0
Salt solution, cc. ....	.4	.4	.6	.7	.7	.8

**Quantitative Serum Test.**—If a quantitative determination of the reagin content of a known positive serum is desired, a series of serum dilutions may be prepared by placing 0.4 cc. of salt solution in each of a series of tubes. To the first tube is added 0.4 cc. of serum, and 0.4 cc. of the resulting mixture is transferred to the following tube: 0.4 cc. is withdrawn from tube 2 and transferred to tube 3, and the process is repeated with all the tubes of the series. The final set-up is then as follows:

Serum control	Test proper							
Serum, cc., 0.2...	0.2	0.1	0.05	0.025	0.0125	0.0062	0.031	
	in a total volume of 0.4 cc. corresponding to a dilution of							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256

It is a technically simpler procedure to prepare a single 1:20 dilution of the serum (0.1 cc. + 1.9 cc. salt solution) and distribute the serum as follows:

Whole serum, cc. ....	0.2	0.05					
Serum, 1:20, cc. ....			0.4	0.2	0.1	0.05	
Salt solution, cc. ....	.2	.35	0.	.2	.3	.35	

**Secondary Incubation of the Tests at 37° C., and the Addition of the Sensitized Cells.**—After 3 to 4 hours at 0° to 5° C., the tests and controls are placed in the 37° C. water bath for 30 minutes. Eight-tenths (0.8) cc. of the sensitized cell suspension is then added to all the tubes, which are vigorously shaken and replaced at 37° C. for their final incubation of 20 to 30 minutes.

This leeway of 10 minutes is allowed the technician to compensate for any slight error in the sensitization of the cell as detected by the complement check just described. If tube 2 of the complement control, which contains half the amount of complement used in the test, is slow to hemolyze, requiring, for example, 30 minutes for complete lysis, then the tests also are given 30 minutes. If, on the other hand, tube 2 shows complete hemolysis in 20 minutes, the results of the tests also should be read in 20 minutes after the addition of the sensitized cells. The futility of any stop watch precision is apparent when we remember that it requires 5 to 15 minutes merely to add cells to a large series of tests.

**Reading of Results.**—(a) The *antigen controls* should be completely hemolyzed. Properly diluted, the antigen fortified with 0.6 or 1 per cent cholesterol is not demonstrably anticomplementary under the conditions of the test. Failure to hemolyze indicates that the complement is defective and is deteriorating under the conditions of the test.

(b) The *complement controls* incubated along with the tests should show approximately the same degree of hemolysis as the first set used to check the amboceptor titration. Any pronounced differences indicate either that the complement is defective

and is deteriorating under the conditions of the test, or that the antigen is significantly anticomplementary under the conditions of the test. Such deterioration with fresh complement indicates that the guinea-pigs are in poor condition, and necessitates the greatest caution in the reading of results.

(c) *Reading of tests proper.*—If the serum as such destroys complement, in the absence of antigen, it is anticomplementary—a result which offers no evidence as to the presence or absence of syphilitic infection. If the serum control is completely hemolyzed, and the tubes of the test containing serum, complement, and antigen show no hemolysis, complement has been fixed by a lipoid-reagin compound, and the result is positive. If all 3 tubes show complete hemolysis, the complement has not been fixed and the result is negative. Finally, if the tests show partial hemolysis, the serum control being completely hemolyzed, only part of the complement has been fixed, and the result is doubtful.

In the quantitative Wassermann test the result is given as the maximum dilution of serum which continues to give a positive result. Similarly, in the spinal fluid test, a Wassermann positive fluid may be said to be positive down to, for example, 0.5 cc.

### KOLMER COMPLEMENT FIXATION TESTS FOR SYPHILIS \*

**Preparation of Antigen.**—A cholesterolized and lecithinized alcoholic extract of heart muscle is employed. Bacto-beef heart, prepared by the Digestive Ferments Company of Detroit, is recommended.

1. Place 30 grams of beef heart powder in a flask with 100 cc. of chemically pure acetone. Stopper tightly. Keep at room temperature for 5 days with brief shaking each day.

2. Filter through fat-free paper or decant and discard the filtrate.

3. Dry the residue and extract with 100 cc. of chemically pure absolute ethyl alcohol in a tightly stoppered flask for 5 days at room temperature, shaking each day.

4. Filter through fat-free paper with slight squeezing of the tissue.

5. Measure the filtrate and add absolute ethyl alcohol to 100 cc.; add 0.2 gm. of cholesterol. The cholesterol is dissolved in 10 cc. of ether and added to the alcoholic filtrate. Shake thoroughly and place in a water bath at 55° C. for 1 hour to aid solution.

6. Allow to stand at room temperature for 2 or 3 days with brief shaking each day. Filter through fat-free paper.

7. Keep the antigen at *room temperature* in a tightly stoppered bottle.

A *new antigen* of increased sensitiveness (*Am. Jour. Clin. Path.* 5: 55, 1935) with practically no change in hemolytic or anticomplementary activity may be prepared in the same manner except that it is reinforced with acetone insoluble lipoids as follows:

(a) Step 1, 2, 3 and 4 as above.

(b) Save the first 4 ether extracts used in the preparation of Kahn or Eagle antigens. Concentrate to about  $\frac{1}{8}$  volume in an evaporating dish and add 3 to 6 volumes

\* For a detailed account of Studies in the Standardization of the Wassermann Reaction upon which these methods are based, including complement fixation tests for bacterial diseases, the identification of blood stains, etc., consult Kolmer's *Serum Diagnosis by Complement Fixation*, Lea and Febiger, Philadelphia; 1928. Antigens and hemolysin may be purchased from the Research Institute of Cutaneous Medicine, 2101 Pine Street, Philadelphia, Pa.

of acetone. After mixing and setting aside overnight, the supernatant acetone is removed and the residue of acetone-insoluble lipoids kept in a refrigerator.

(c) Measure the alcoholic filtrate, add absolute ethyl alcohol to 100 cc. and for each cubic centimeter add 0.002 gram of cholesterol. Dissolve the cholesterol and 1 to 2 grams of the acetone-insoluble lipoids in 20 cc. of ether and add to the alcoholic extract in a tightly stoppered bottle or flask.

(d) Shake thoroughly and place in a water bath at 55° C. for 1 hour to aid in the solution of the lipoids.

(e) Allow to stand at room temperature for 2 or 3 days with brief shaking each day. Filter through fat-free paper.

(f) Keep at *room temperature*. Do not disturb any sediment that may be present.

**Hemolytic System and Its Adjustment.**—*Sheep Corpuscle Suspension.*—1. Read the volume of sheep cells in the centrifuge tube after the last washing, carefully remove the supernatant fluid and prepare a 2 per cent suspension by washing the corpuscles into a flask with 49 volumes of saline solution. Otherwise 2 cc. of packed corpuscles may be removed with a pipet and diluted with 98 cc. saline solution.

2. Keep in a refrigerator when not being used. *Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when not in use.*

3. The dose is 0.5 cc. of the 2 per cent suspension.

**Titration of Hemolysin.**—It is advisable (but not absolutely necessary) to make this titration each time the tests are conducted.

1. Prepare a stock dilution of 1:100 hemolysin by mixing 2.0 cc. of glycerinized hemolysin (50 per cent) with 94.0 cc. saline solution and 4.0 cc. of 5 per cent phenol in saline solution. This will keep in a refrigerator for several months.

2. Prepare a 1:1000 dilution by mixing 0.5 cc. of 1:100 with 4.5 cc. saline solution. In a series of 10 test tubes prepare higher dilutions (with thorough mixing) as follows:

No.	1.	0.5 cc. hemolysin (1:1000) = 1:1000
No.	2.	0.5 cc. hemolysin (1:1000) + 0.5 cc. saline solution = 1:2000
No.	3.	0.5 cc. hemolysin (1:1000) + 1.0 cc. saline solution = 1:3000
No.	4.	0.5 cc. hemolysin (1:1000) + 1.5 cc. saline solution = 1:4000
No.	5.	0.5 cc. hemolysin (1:1000) + 2.0 cc. saline solution = 1:5000
No.	6.	0.5 cc. hemolysin (1:3000) + 0.5 cc. saline solution = 1:6000
No.	7.	0.5 cc. hemolysin (1:4000) + 0.5 cc. saline solution = 1:8000
No.	8.	0.5 cc. hemolysin (1:5000) + 0.5 cc. saline solution = 1:10,000
No.	9.	0.5 cc. hemolysin (1:6000) + 0.5 cc. saline solution = 1:12,000
No.	10.	0.5 cc. hemolysin (1:8000) + 0.5 cc. saline solution = 1:16,000

3. Prepare 1:30 dilution of complement for hemolysin and complement titrations by diluting 0.2 cc. of complement serum with 5.8 cc. of saline solution. Prepare a 2 per cent suspension of sheep corpuscles.

4. In a series of 10 tubes set up the hemolysin titration as shown in Table 35A.

5. Mix the contents of each tube and incubate in the water bath at 37° C. for 1 hour. Read the unit of hemolysin. *The unit is the highest dilution of hemolysin that gives complete sparkling hemolysis.*

6. Two units are used in the complement and antigen titrations and in the com-

plement fixation tests. Hemolysin is so diluted that 0.5 cc. contains 2 units. For example, if the unit equals 0.5 cc. of 1:6000, 2 units equals 0.5 cc. of 1:3000. Dilute just enough hemolysin for the complement titration and the complement fixation tests. *Keep the hemolysin in the refrigerator when not in use.*

TABLE 35-A—HEMOLYSIN TITRATION

Tube	Hemolysin, 0.5 cc.	Complement, cc. (1:30)	Saline Solution, cc.	Corpuscles, cc.
1	1:1000	0.3	1.7	0.5
2	1:2000	0.3	1.7	0.5
3	1:3000	0.3	1.7	0.5
4	1:4000	0.3	1.7	0.5
5	1:5000	0.3	1.7	0.5
6	1:6000	0.3	1.7	0.5
7	1:8000	0.3	1.7	0.5
8	1:10,000	0.3	1.7	0.5
9	1:12,000	0.3	1.7	0.5
10	1:16,000	0.3	1.7	0.5

7. The following table shows how the dilutions are made so that 0.5 cc. carries 2 units:

1 Unit 0.5 cc. of	2 Units Would Be 0.5 cc. of	Prepared by Diluting 1 cc. of Stock 1:100 with Following Amounts of Saline
1:1000	1:500	4 cc.
1:2000	1:1000	9 cc.
1:3000	1:1500	14 cc.
1:4000	1:2000	19 cc.
1:5000	1:2500	24 cc.
1:6000	1:3000	29 cc.
1:8000	1:4000	39 cc.
1:10,000	1:5000	49 cc.

8. High titer hemolysin is recommended and the unit should be 0.5 cc. of 1:4000 or higher.

9. In practice the hemolysin titration may be placed in the water bath at the same time as the complement titration; at the end of the first incubation of the complement titration the unit of hemolysin is available and 2 units added to all tubes of the complement titration, etc.

*Pretesting of Complement.*—When conditions permit it is advisable (but not absolutely necessary) to test the complement serum of each individual guinea-pig before satisfactory complement sera are pooled for the complement titration and complement fixation tests. The technic for testing *each serum* is as follows:

1. Prepare a 1:30 dilution by mixing 0.15 cc. with 4.35 cc. saline solution.
2. In a series of 6 test tubes place 0.8, 0.6, 0.4, 0.8, 0.6 and 0.4 cc. respectively.
3. To each of the first 3 tubes add 0.5 cc. of the *same antigen to be used in the*

*complement titration and the complement fixation tests* so diluted as to carry the optimum dose.

4. Add saline solution to a total volume of 2.0 cc. in each tube.
5. Mix and place in a refrigerator at 6° to 8° C. for 15 to 18 hours, or overnight, followed by 10 minutes in a water bath at 37° C.
6. Add 0.5 cc. of hemolysin carrying 2 units and 0.5 cc. of 2 per cent suspension of sheep cells to each tube.
7. Mix and place in a water bath at 37° C. for 1 hour and examine.
8. Sera showing complete and sparkling hemolysis in all 6 tubes are entirely satisfactory. Sera showing an equal degree of inhibition of hemolysis in the first 3 tubes carrying antigen as the last 3 tubes carrying no antigen may be used but will usually be found to have a low unit of hemolytic activity in the complement titration described below. Any serum showing a greater degree of inhibition of hemolysis in the first 3 tubes carrying antigen than in the last 3 tubes carrying no antigen should be regarded as unsatisfactory for the complement titration and complement fixation tests insofar as the particular antigen employed is concerned. Such sera may be satisfactory with another Kolmer antigen.

*Titration of Complement.*—1. Prepare a 1:30 dilution of complement in saline solution.

2. Dilute antigen so that the dose employed in the main tests is contained in 0.5 cc. This dilution is prepared by placing the required amount of saline solution in a flask and adding the required amount of antigen drop by drop. Shake the flask after each addition of antigen. Prepare enough diluted antigen for the complement titration and the complement fixation tests.

3. In a series of 8 test tubes set up the titration as shown in Table 36.

**TABLE 36.—COMPLEMENT TITRATION**

Tube	Complement, cc. (1:30)	Antigen, Dose cc.	Saline Solution, cc.	Water bath 37° C. for one hour	Hemolysin, cc. (2 units)	Corpuscles, cc. (2 per cent)	Water bath 37° C. for one hour
1	0.2	0.5	1.3		0.5	0.5	
2	0.25	0.5	1.3		0.5	0.5	
3	0.3	0.5	1.2		0.5	0.5	
4	0.35	0.5	1.2		0.5	0.5	
5	0.4	0.5	1.1		0.5	0.5	
6	0.45	0.5	1.1		0.5	0.5	
7	0.5	0.5	1.0		0.5	0.5	
8	None	None	2.5		None	0.5	

4. The smallest amount of complement just giving complete sparkling hemolysis is the *exact unit*. The next higher tube is the *full unit* which contains 0.05 cc. more complement. In conducting the antigen titration and complement fixation tests, *two full units* are employed and so diluted as to be contained in 1 cc. as per the following example:

Exact unit: 0.3 cc.

Full unit: 0.35 cc.

Dose (two full units): 0.7 cc.



To calculate the dilution to employ so that 1 cc. contains the dose of 2 full units, divide 30 by the dose:

$$\frac{30}{0.7} = 43 \text{ or } 1 \text{ cc. of } 1:43 \text{ dilution of serum.}$$

The following table gives additional examples:

Exact Unit (cc.)	Full Unit (cc.)	Two Full Units (cc.)	Dilution to use	Preparation
0.2	0.25	0.5	1:60	1 cc. serum + 59 cc. saline
0.25	0.3	0.6	1:50	1 cc. serum + 49 cc. saline
0.3	0.35	0.7	1:43	1 cc. serum + 42 cc. saline
0.35	0.4	0.8	1:37	1 cc. serum + 36 cc. saline
0.4	0.45	0.9	1:33	1 cc. serum + 32 cc. saline
0.45	0.5	1.0	1:30	1 cc. serum + 29 cc. saline
0.5	0.55	1.1	1:27	1 cc. serum + 26 cc. saline

5. If however the complement is unusually sensitive to the anticomplementary effects of antigen and serum in the conduct of the complement fixation tests, as is sometimes the case during the hot summer months,  $2\frac{1}{2}$  exact units may be employed and so diluted that 1 cc. contains this dose. Example:

Exact unit: 0.35 cc.

$2\frac{1}{2}$  units: 0.88 cc.

To calculate the dilution to use so that 1 cc. contains the dose, divide 30 by the dose:

$$\frac{30}{0.88} = 34 \text{ or } 1 \text{ cc. of } 1:34 \text{ dilution of serum.}$$

The following table gives additional examples:

Unit (cc.)	$2\frac{1}{2}$ Units (cc.)	Dilution to Use	Preparation
0.2	0.5	1:60	1 cc. serum + 59 cc. saline
0.25	0.63	1:47	1 cc. serum + 46 cc. saline
0.3	0.75	1:40	1 cc. serum + 39 cc. saline
0.35	0.88	1:34	1 cc. serum + 33 cc. saline
0.4	1.0	1:30	1 cc. serum + 29 cc. saline
0.45	1.13	1:27	1 cc. serum + 26 cc. saline
0.5	1.25	1:24	1 cc. serum + 23 cc. saline

6. *It is always advisable to dilute complement serum with cold saline solution instead of with saline kept at room temperature. Undiluted and especially diluted complement serum should always be kept in a refrigerator when not in use.* Exposure of diluted complement to room temperature for over an hour may result in some deterioration.

7. Occasionally hyperactive complement yields a unit of 0.2 to 0.25 cc. of 1:30 but when this occurs it is necessary to arbitrarily take 0.3 cc. as the exact unit as less complement falls below the absolute minimum and is likely to be unsatisfactory.

**Titration of Antigen.**—It is not necessary to titrate for hemolytic and anti-complementary units as hitherto advised because titrations of over 600 antigens by Kolmer during the past 20 years have never shown any to be hemolytic in 0.5 cc. of 1:4 and the anticomplementary units have been uniformly from 0.5 cc. of 1:6 to not higher than 1:10. Therefore, these titrations may be omitted providing the antigen is prepared by either of the methods previously described.

It is necessary, however, to titrate for antigenic activity and the following method after that of Boerner and Lukens (*Am. Jour. Clin. Path.* 7: 33, 1937) for determining the optimum dose, as based upon principles previously described by Hooker (*Jour. Immunol.*, 14: 129, 1927), is as follows:

1. Prepare a 1:80 dilution of antigen by adding 0.1 cc., drop by drop with shaking between each, to 7.9 cc. of saline solution in a large test tube or small flask. Higher dilutions are then prepared as follows:

4 cc. of 1:80 + 4 cc. saline solution = 1:160  
 4 cc. of 1:160 + 4 cc. saline solution = 1:320  
 4 cc. of 1:320 + 4 cc. saline solution = 1:640  
 4 cc. of 1:640 + 4 cc. saline solution = 1:1280  
 4 cc. of 1:1280 + 4 cc. saline solution = 1:2560

2. Arrange 5 rows of test tubes with 6 in each row. In the *first* tube of each row place 0.5 cc. of antigen 1:80; to the *second* tube of each row 0.5 cc. of antigen 1:160; to the *third* tube, 0.5 cc. of 1:320; to the *fourth*, 0.5 cc. of 1:640; to the *fifth* 0.5 cc. of 1:1280 and to the *sixth* 0.5 cc. of 1:2560.

3. Heat 3 cc. of a mixture of positive syphilitic sera in a water bath at 55° C. for 15 to 20 minutes and prepare 5 dilutions as follows in large test tubes:

1.0 cc. serum + 4.0 cc. saline = 1:5 (0.5 cc. carries 0.1 cc. serum)  
 0.5 cc. serum + 4.5 cc. saline = 1:10 (0.5 cc. carries 0.05 cc. serum)  
 0.5 cc. serum + 9.5 cc. saline = 1:20 (0.5 cc. carries 0.025 cc. serum)  
 2.0 cc. serum 1:20 + 2.0 cc. saline = 1:40 (0.5 cc. carries 0.0125 cc. serum)  
 1.0 cc. serum 1:20 + 4.0 cc. saline = 1:100 (0.5 cc. carries 0.005 cc. serum)

4. Add 0.5 cc. of 1:5 dilution to each of the 6 tubes of the first row; 0.5 cc. of 1:10 to each tube of the second row; 0.5 cc. of 1:20 to each tube of the third row; 0.5 cc. of 1:40 to each tube of the fourth row and 0.5 cc. of 1:100 to each tube of the fifth row.

5. Add 1 cc. of complement dilution carrying 2 full units to all tubes.

6. Put up a *serum control* carrying 0.5 cc. of 1:5 serum and 1 cc. of complement (2 full units); also a *hemolytic system control* carrying 1 cc. of saline solution and 1 cc. of complement (2 full units).

7. Shake the tubes gently and place in refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath at 37° C. for 10 minutes.

8. Add 0.5 cc. of hemolysin (2 units) and 0.5 cc. of 2 per cent suspension of corpuscles to all tubes.

9. Mix thoroughly and place in a water bath at 37° C. for one hour; make readings. The serum and hemolytic system controls should show complete hemolysis.

10. Chart the results as per the following example observed with a strongly positive serum:

Serum in 0.5 cc.	Antigen in 0.5 cc. amounts					
	1:80	1:160	1:320	1:640	1:1280	1:2560
0.005 .....	—	—	++	—	—	—
0.0125 .....	—	+	++++	++++	++	+
0.025 .....	+	++++	++++	++++	++++	+
0.05 .....	+++	++++	++++	++++	++++	++
0.1 .....	++++	++++	++++	++++	++++	+++

11. The optimum dose of antigen to employ in the main tests is the *amount giving a ++++ reaction with the smallest amount of serum*. If 2 dilutions of antigen give ++++ reactions with the smallest amount of serum, the dose may be the average of the 2 as, for example, 0.5 cc. of 1:500 in the above table instead of 0.5 cc. of 1:490 in order to simplify its preparation. If 3 dilutions of antigen give ++++ reactions with the smallest amount of serum, the optimum dose is midway between the highest and lowest. Before a new antigen is used, however, it is always advisable to check the antigenic sensitivity of the optimum dose against an antigen of proven sensitivity in a series of comparative tests employing weakly to moderately positive syphilitic sera.

**Choice of Methods.**—The *regular quantitative method* employs 5 doses of serum or spinal fluid and is preferred when conditions permit, especially in testing the sera and spinal fluids of cases of syphilis under treatment. The *regular simplified method* employs one dose of serum (0.2 cc.) or spinal fluid (0.5 cc.) and is satisfactory for routine diagnostic purposes as well as being more economical of time and reagents when large numbers of tests are being conducted. Otherwise the simplified test may be conducted with 2 doses of serum (0.2 and 0.1 cc.) as a safeguard against prezone reactions, in which the second tube carrying 0.1 cc. may give a stronger reaction than the first tube carrying 0.2 cc. of serum.

In the interests of economy both tests may be conducted by using the reagents in *one-half* or *one-fifth* amounts. This economy applies not only to the amounts of serum and spinal fluid but to the amounts of complement, antigen, hemolysin and sheep corpuscles employed. Thus the amount of complement required for  $\frac{1}{5}$  methods is about 3 times less than for the regular tests with the result that about 2 cc. is sufficient for the conduct of 100 simplified tests.

**Use of Egg Albumin.**—As shown by Boerner and Lukens (*Am. Jour. Clin. Path.* 11: 71, 1941), the addition of 0.2 cc. of a 50 per cent solution of egg albumin in sterile saline solution to each tube of the regular quantitative and simplified tests employing *spinal fluid* is advisable for the prevention of prezone and nonspecific reactions. Kolmer and Lynch (*Am. Jour. Clin. Path.* 11: 402, 1941) have confirmed these observations and advise the addition of egg albumin routinely in the conduct of quantitative and simplified tests with spinal fluids although it may be omitted if pretested complement is being employed and if prezone reactions are not being observed. They also advise its use in the conduct of quantitative serum tests if and when prezone reactions with normal sera (like — — 1 2 2) are being observed. It is not necessary, however, to use egg albumin in the simplified serum tests.

The egg albumin solution may be prepared as follows: (a) Break a fresh egg and

separate the white from the yolk; (b) pick out heavy particles or filter through several layers of gauze; (c) measure, beat briefly and add an equal volume of sterile saline solution; (d) keep in a refrigerator (may be used over a week or two without the addition of a preservative). An alternate method is to prepare a 10 per cent solution in sterile saline solution and to use this for diluting the complement so that 1 cc. carries 2 full units. This 10 per cent solution may be prepared by diluting 10 cc. of albumin with 90 cc. of sterile saline solution or by diluting 20 cc. of the 50 per cent solution with 80 cc. of sterile saline solution.

**Regular Quantitative Tests.**—1. *For each serum:* (a) Remove the natural anti-sheep hemolysin as described on page 664 and heat in a water bath at 55° to 56° C. for 15 to 20 minutes.

(b) Arrange 6 test tubes (85 x 13 mm. inside diameter) and place the following amounts of saline solution respectively: 0.9, 0.5, 0.5, 0.5, 2.0 and 0.5 cc. Add 0.6 cc. serum to No. 1, mix by drawing up in the tube several times and transfer 0.5 cc. to No. 2 and No. 6. Mix No. 2 and transfer 0.5 cc. to No. 3 and so on to No. 5 from which discard 2.0 cc. after mixing. This leaves 0.5 cc. in each of the first 5 tubes carrying 0.2, 0.1, 0.05, 0.025 and 0.005 cc. serum respectively. Tube No. 6 (serum control) carries 1.0 cc. (0.2 cc. serum) since it receives no antigen and thereby making the total volume in all tubes the same when the test is completed. In case prezone reactions are being observed, as may be the case if the complement is not pretested, add 0.2 cc. of a 50 per cent solution of egg albumin to each of the six tubes and the antigen control.

(c) Complete the test including antigen, hemolytic system and corpuscle controls as shown in Table 37. It is advisable to include tests with known positive and negative sera as controls.

2. *For each spinal fluid:* (a) Arrange 6 test tubes and place 0.5 cc. of saline solution in Nos. 2, 3, 4, 5 and 6.

(b) Add 0.5 cc. of spinal fluid to tubes 1, 2 and 6. Mix No. 2 and transfer 0.5 cc. to No. 3 and so on to No. 5 from which discard 0.5 cc. after mixing. Tubes 1 to 5 now contain 0.5 cc. carrying 0.5, 0.25, 0.125, 0.0625 and 0.03125 cc. of spinal fluid. Tube No. 6 (control) contains 1 cc. carrying 0.5 cc. of spinal fluid.

(c) Add 0.2 cc. of 50 per cent solution of egg albumin in saline solution to each of the 6 tubes and the antigen control. (Alternate method, diluting complement with 10 per cent egg albumin in saline, may be used.)

(d) Complete the test with antigen, hemolytic system and corpuscle controls as shown in Table 38. It is advisable to include tests with known positive and negative spinal fluids as controls.

3. During the secondary incubation watch the *serum or spinal fluid, antigen, and hemolytic system controls and 10 minutes after these show complete hemolysis (usually 25 to 30 minutes) remove the tests and make the readings.* In the case of those serum or spinal fluid controls in which hemolysis is incomplete, continue the incubation for a total of one hour which frequently permits the reading of tests with sera and spinal fluids that are slightly anticomplementary.

**Regular Simplified Tests.**—1. *For each serum:* (a) The removal of natural anti-sheep hemolysin may be omitted. Heat the sera in a water bath at 55° to 56° C. for 15 to 20 minutes although heating for 30 minutes is permissible if flocculation tests are to be conducted.

**TABLE 37.—REGULAR QUANTITATIVE SERUM TEST**

Tube	Serum (in 0.5 cc.)	Antigen cc.	Interval of 10-30 min. at room temperature *			Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 10-15 min. in water bath at 37° C.			Mix all tubes. Secondary incubation in water bath at 37° C.		
				Complement cc. (2 full units)	Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)					
1	0.2 cc.	0.5		1.0	0.5	0.5					
2	0.1 cc.	0.5		1.0	0.5	0.5					
3	0.05 cc.	0.5		1.0	0.5	0.5					
4	0.025 cc.	0.5		1.0	0.5	0.5					
5	0.005 cc.	0.5		1.0	0.5	0.5					
6	0.2 cc. (Cont)	none		1.0	0.5	0.5					
7	Antigen Control: 0.5 cc. saline	0.5		1.0	0.5	0.5					
8	Hemolytic Cont: 1.0 cc. saline	none		1.0	0.5	0.5					
9	Corpuscle Cont: 2.5 cc. saline	none		none	none	0.5					

\* If a longer time elapses place the tubes in a refrigerator.

**TABLE 38.—REGULAR QUANTITATIVE SPINAL FLUID TEST**

Tube	Spinal Fluid (in 0.5 cc.)	Egg * Albumin (50%) cc.	Antigen cc.	Interval of 10-30 min. at room temperature **		Complement cc. (2 full units)	Mix all tubes. Primary incubation 6-8° C. for 15-18 hours followed by 10-15 min. in water bath at 37° C.		Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)	Mix all tubes. Secondary incubation in water bath at 37° C.
1	0.5 cc.	0.2	0.5			1.0			0.5	0.5	
2	0.25 cc.	0.2	0.5			1.0			0.5	0.5	
3	0.125 cc.	0.2	0.5			1.0			0.5	0.5	
4	0.0625 cc.	0.2	0.5			1.0			0.5	0.5	
5	0.03125 cc.	0.2	0.5			1.0			0.5	0.5	
6	0.5 cc. (Cont.)	0.2	none			1.0			0.5	0.5	
7	Antigen Control: 0.5 cc. saline	0.2	0.5			1.0			0.5	0.5	
8	Hemolytic Cont.: 1.0 cc. saline	none	none			1.0			0.5	0.5	
9	Corpuscle Cont.: 2.5 cc. saline	none	none			none			none	0.5	

\* Can usually be omitted if pretested complement is employed.

\*\* If a longer time elapses place the tubes in a refrigerator.

**TABLE 39.—REGULAR SIMPLIFIED SERUM TEST**

Tube	Serum (in 0.5 cc.)	Antigen cc.	Interval of 10-30 min. at room temperature *		Complement cc. (2 full units)	Mix all tubes. Primary in- cubation 6°-8° C. for 15-18 hours followed by 10-15 min. in water bath 37° C.		Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)	Mix all tubes. Secondary in- cubation in water bath at 37° C.
1	0.2 cc.	0.5			1.0			0.5		
2	0.2 cc. (Cont)	None			1.0			0.5		
3	Antigen Control: 0.2 cc. saline	0.5			1.0			0.5		
4	Hemolytic Cont: 1.0 cc. saline	None			1.0			0.5		
5	Corpuscle Cont: 2.5 cc. saline	None			None			None	0.5	

\* If a longer time elapses place the tubes in a refrigerator.

(b) Arrange 2 test tubes and place 0.5 cc. of saline solution in No. 2. Add 0.2 cc. of serum to each tube.

(c) Complete the test including antigen, hemolytic system and corpuscle controls as shown in Table 39. It is advisable to include tests with known positive and negative sera as controls.

(d) If it is desired to conduct the test with 0.2 and 0.1 cc. amounts of serum arrange 3 test tubes and place 0.9, 0.5 and 0.5 cc. saline solution in each respectively. Add 0.6 cc. of serum to No. 1, mix and transfer 0.5 cc. to tubes 2 and 3 (control). Each tube now carries 0.5 cc. containing 0.2, 0.1 and 0.2 cc. serum respectively. Antigen (0.5 cc.) is added to Nos. 1 and 2 and the test completed as shown in Table 39.

2. *For each spinal fluid:* (a) Arrange 2 test tubes and place 0.5 cc. saline solution in No. 2. Add 0.5 cc. spinal fluid to each tube. Add 0.2 cc. of 50 per cent solution of egg albumin in saline solution to each tube and the antigen control.

(b) Complete the test including antigen, hemolytic system and corpuscle controls as shown in Table 40. It is advisable to include tests with known positive and negative spinal fluids as controls.

3. During the secondary incubation *watch the serum or spinal fluid, antigen, and hemolytic system controls and 10 minutes after these show complete hemolysis (usually 25 to 30 minutes) remove the tests and make the readings.* In the case of those serum and spinal fluid controls in which hemolysis is incomplete, continue the incubation for a total of 1 hour which frequently permits the reading of tests with sera and spinal fluids that are slightly anticomplementary.

**One-Half Methods.**—The hemolysin and complement are titrated and diluted in the same manner as in the regular methods. The dose of the former carrying 2 units is 0.25 instead of 0.5 cc. and the dose of the latter carrying 2 full units is 0.5 instead of 1.0 cc. The dose of 2 per cent corpuscle suspension is 0.25 instead of 0.5 cc.

1. *For each quantitative serum test:* (a) Arrange 6 test tubes and place the following amounts of saline solution respectively: 0.6, 0.3, 0.3, 0.3, 1.2 and 0.3 cc. (b) Add 0.3 cc. serum to No. 1, mix and transfer 0.3 cc. to No. 2 and No. 6. Mix No. 2 and transfer 0.3 cc. to No. 3 and so on to No. 5 from which discard 1.2 cc. after mixing. In case prezone reactions are being observed, as may be the case if the complement is not pretested, add 0.1 cc. of 50 per cent solution to each of the 6 tubes and the antigen control. (c) Complete the test including antigen, hemolytic system and corpuscle controls as shown in Table 41. It is advisable to include tests with known positive and negative sera as controls.

2. *For each quantitative spinal fluid test:* (a) Arrange 6 test tubes and place 0.25 cc. saline solution in Nos. 2, 3, 4, 5 and 6. (b) Add 0.25 cc. of spinal fluid to tubes 1, 2 and 6. (c) Mix No. 2 and transfer 0.25 cc. to No. 3 and so on to No. 5 from which discard 0.25 cc. after mixing. (d) Add 0.1 cc. of 50 per cent solution of egg albumin to all tubes including the antigen control. (e) Complete the test with antigen, hemolytic system and corpuscle controls as shown in Table 42. It is advisable to include tests with known positive and negative spinal fluids as controls.

3. The secondary incubation is conducted and the reactions read as described above for the regular methods.

**One-Fifth Methods.**—These are conducted in exactly the same manner as the regular quantitative and simplified serum and spinal fluid tests except that smaller



TABLE 40.—REGULAR SIMPLIFIED SPINAL FLUID TEST

Tube	Spinal Fluid (in 0.5 cc.)	Egg* Albumin (50%) cc.	Antigen cc.	Interval of 10-30 min. at room temperature**		Complement cc. (2 full units)	Mix all tubes. Primary in- cubation 6-8° C. for 15- 18 hours followed by 10- 15 min. in water bath at 37° C.		Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)	Mix all tubes. Secondary incubation in water bath at 37° C.
1	0.5 cc.	0.2	0.5			1.0			0.5	0.5	
2	0.5 cc. (Cont)	0.2	None			1.0			0.5	0.5	
3	Antigen Control 0.5 cc. saline	0.2	0.5			1.0			0.5	0.5	
4	Hemolytic Cont: 1.0 cc. saline	None	None			1.0			0.5	0.5	
5	Corpuscle Cont: 2.5 cc. saline	None	None			None			None	0.5	

\* Can usually be omitted if pretested complement is employed.

\*\* If a longer time elapses place the tubes in a refrigerator.

**TABLE 41.—ONE-HALF QUANTITATIVE SERUM TEST**

Tube	Serum (in 0.3 cc.)	Antigen cc.	Interval of 10-30 min. at room temperature *			Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 10-15 min. in water bath at 37° C.			Mix all tubes. Secondary incubation in water bath at 37° C.		
1	0.1 cc.	0.25	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25
2	0.05 cc.	0.25									
3	0.025 cc.	0.25									
4	0.0125 cc.	0.25									
5	0.0025 cc.	0.25									
6	0.1 cc. (Cont.)	0.25									
7	Antigen Control: 0.5 cc. saline	None	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25
8	Hemolytic Cont: 0.5 cc. saline	None									
9	Corpuscle Cont: 1.3 cc. saline	None									

\* If a longer time elapses place the tubes in a refrigerator.

**TABLE 42.—ONE-HALF QUANTITATIVE SPINAL FLUID TEST**

Tube	Spinal Fluid (in 0.25 cc.)	Egg * Albumin (50%) cc.	Antigen cc.	Interval of 10-30 min. at room temperature **		Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 10-15 min. in water bath at 37° C.		Hemolysin (2 units) cc.	Corpuscles (2 per cent) cc.	Mix all tubes. Secondary incubation in water bath at 37° C.	
1	0.25 cc.	0.1	0.25			0.5		0.25	0.25		
2	0.125 cc.	0.1	0.25			0.5		0.25	0.25		
3	0.0625 cc.	0.1	0.25			0.5		0.25	0.25		
4	0.03125 cc.	0.1	0.25			0.5		0.25	0.25		
5	0.015625 cc.	0.1	0.25			0.5		0.25	0.25		
6	0.25 (Control)	0.1	0.25			0.5		0.25	0.25		
7	Antigen Control: 0.5 cc. saline	0.1	None			0.5		0.25	0.25		
8	Hemolytic Cont.: 0.5 cc. saline	None	None			0.5		0.25	0.25		
9	Corpuscle Cont.: 1.3 cc. saline	None	None			None		None	0.25		

\* Can usually be omitted if pretested complement is employed.

\*\* If a longer time elapses place the tubes in a refrigerator.

test tubes (75 by 10 mm. inside diameter) and one-fifth amounts of the various reagents are employed.

1. The *hemolysin* is titrated in the same manner using 0.1 cc. of the varying dilutions (1:1000 to 1:16000), 0.1 cc. of 1:50 complement and 0.1 cc. of 2 per cent suspension of sheep corpuscles; add 0.4 cc. saline solution to each tube. Two units are used in the complement titration and main tests. For example, if the unit is 0.1 cc. of 1:6000, 2 units equal 0.1 cc. of 1:3000.

2. For *pretesting complement*: (a) Dilute 0.1 cc. of each guinea-pig serum with 4.9 cc. saline solution (1:50). (b) Place 0.4, 0.3, 0.2, 0.4, 0.3 and 0.2 cc. in each of 6 test tubes respectively. (c) To each of the first 3 tubes add 0.1 cc. of antigen of the same dilution as employed in the regular tests. (d) Add saline to tubes 2, 3, 4, 5 and 6 to make the total volume 0.6 cc. in each. (e) Mix and place in refrigerator at 6° to 8° C. for 15 to 18 hours, or overnight, followed by 10 minutes in a water bath at 37° C. (f) Add 0.1 cc. of hemolysin (2 units) and 0.1 cc. of 2 per cent corpuscle suspension to all tubes. (g) Mix and incubate in water bath at 37° C. for 1 hour and read. Complement sera showing complete and sparkling hemolysis in all tubes are satisfactory; those showing the same degree of inhibition of hemolysis in the first 3 tubes carrying antigen as the last 3 carrying no antigen may be employed. Complement sera showing a greater degree of inhibition of hemolysis in the first 3 tubes are unsatisfactory insofar as the particular antigen employed is concerned; they may be satisfactory with another antigen.

3. The *complement titration* is conducted (a) by placing 0.1, 0.15, 0.2, 0.25 and 0.3 cc. of 1:50 dilution in each of 5 test tubes respectively. (b) To each tube add 0.1 cc. of antigen of the same dilution as employed in the regular tests. (c) Add 0.3, 0.3, 0.2, 0.2 and 0.1 cc. of saline solution to the tubes respectively. (d) Mix and place in water bath at 37° C. for 1 hour. (e) Add 0.1 cc. hemolysin (2 units) and 0.1 cc. of 2 per cent corpuscle suspension to all tubes. (f) Mix, place in water bath at 37° C. for 1 hour and read. The *exact unit* is the smallest amount giving complete sparkling hemolysis. For conducting the tests *two exact units* are employed instead of 2 full units. This dose of 2 exact units should be contained in 0.2 cc. of the proper dilution prepared as follows:

<i>Exact Unit</i>	<i>Two Exact Units</i>	<i>Dose of Two Exact Units</i>
0.10 cc.	0.2 cc.	0.2 cc. of 1:50 dilution
0.15 cc.	0.3 cc.	0.2 cc. of 1:33 dilution
0.20 cc.	0.4 cc.	0.2 cc. of 1:25 dilution
0.25 cc.	0.5 cc.	0.2 cc. of 1:20 dilution
0.30 cc.	0.6 cc.	0.2 cc. of 1:17 dilution

4. For each *quantitative serum test*: (a) Arrange 6 test tubes and place 0.8, 0.2, 0.2, 0.2, 0.8 and 0.1 cc. saline solution respectively. (b) To tube No. 1 add 0.2 cc. inactivated serum; mix and transfer 0.2 cc. to tubes 2 and 6; discard 0.4 cc. (c) Mix No. 2 and transfer 0.2 cc. to No. 3 and so on to No. 5 from which discard 0.8 cc. after mixing. This leaves 0.2 cc. in each tube carrying 0.04, 0.02, 0.01, 0.005, 0.001 and 0.04 cc. (control) respectively. (d) If prezone reactions are being observed, add 0.1 cc. of a 20 per cent solution of egg albumin to each tube including the antigen control. (e) Complete the tests including antigen, hemolytic system and corpuscle controls, as shown in Table 43; it is advisable to include tests with positive and negative sera.

**TABLE 43.—ONE-FIFTH QUANTITATIVE SERUM TEST**

Tube	Serum (in 0.2 cc.)	Antigen cc.	Interval of 10-30 min. at room temperature *			Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 10-15 min. in water bath at 37° C.			Mix all tubes. Secondary incubation in water bath at 37° C.		
1	0.04 cc.	0.1			Complement cc. (2 exact units)				Hemolysin cc. (2 units)		Corpuscles cc. (2 per cent)
2	0.02 cc.	0.1			0.2				0.1		0.1
3	0.01 cc.	0.1			0.2				0.1		0.1
4	0.005 cc.	0.1			0.2				0.1		0.1
5	0.001 cc.	0.1			0.2				0.1		0.1
6	0.04 cc. (control)	None			0.2				0.1		0.1
7	Antigen control 0.2 cc. saline sol.	0.1			0.2				0.1		0.1
8	Hemolytic control 0.3 cc. saline sol.	None			0.2				0.1		0.1
9	Corpuscle control 0.6 cc. saline sol.	None			None				None		0.1

\* If a longer time elapses place the tubes in a refrigerator.

**TABLE 44.—ONE-FIFTH SIMPLIFIED SERUM TEST**

Tube	Serum (in 0.2 cc.)	Antigen cc.	Interval of 10-15 min. at room temp.*	Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 10-15 min. in water bath at 37° C.			Mix all tubes. Secondary incubation in water bath at 37° C.		
				Complement cc. (2 exact units)	Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)			
1	0.04 cc.	0.1		0.2	0.1	0.1			
2	0.04 cc. (control)	0.1		0.2	0.1	0.1			
3	Antigen control 0.3 cc. saline sol.	None		0.2	0.1	0.1			
4	Hemolytic control 0.3 cc. saline sol.	None		0.2	0.1	0.1			
5	Corpuscle control 0.6 cc. saline sol.	None		None	None	0.1			

\* If a longer time elapses place the tubes in a refrigerator.

5. For each *quantitative spinal fluid test*: (a) Arrange 6 test tubes and place 0.3, 0.2, 0.2, 0.2 and 0.2 cc. saline in the first 5 respectively. (b) To tube No. 1 add 0.3 cc. spinal fluid, mix and transfer 0.2 cc. to No. 2 and No. 6. (c) Mix No. 2 and transfer 0.2 cc. to No. 3 and so on to No. 5 from which discard 0.2 cc. after mixing. This leaves 0.2 cc. in each tube carrying 0.1, 0.05, 0.025, 0.0125, 0.00625 and 0.1 cc. (control) of spinal fluid respectively. (d) To each tube, including the antigen control, add 0.2 cc. of a 10 per cent solution of egg albumin in saline solution. (e) Complete the test including antigen, hemolytic system and corpuscle controls as shown in Table 43; it is advisable to include tests with known positive and negative spinal fluids as controls.

6. For each *simplified serum test*: (a) Arrange 2 test tubes and place 0.8 cc. of saline solution in No. 1. (b) Add 0.2 cc. of inactivated serum to No. 1; mix, transfer 0.2 cc. to No. 2 and discard 0.6 cc. This leaves 0.04 cc. serum in each of the 2 tubes (No. 2 being the serum control). (c) Complete the tests including antigen, hemolytic system and corpuscle controls, as shown in Table 44; it is advisable to include tests with known positive and negative sera as controls.

7. For each *simplified spinal fluid test*: (a) Arrange 2 test tubes and place 0.1 cc. saline solution in No. 1 and 0.2 cc. in No. 2. (b) Add 0.1 cc. spinal fluid to each. (c) To each tube and the antigen control add 0.2 cc. of 10 per cent solution of egg albumin in saline solution. (d) Complete the test including antigen, hemolytic system and corpuscle controls as shown in Table 44; it is advisable to include tests with known positive and negative spinal fluids as controls.

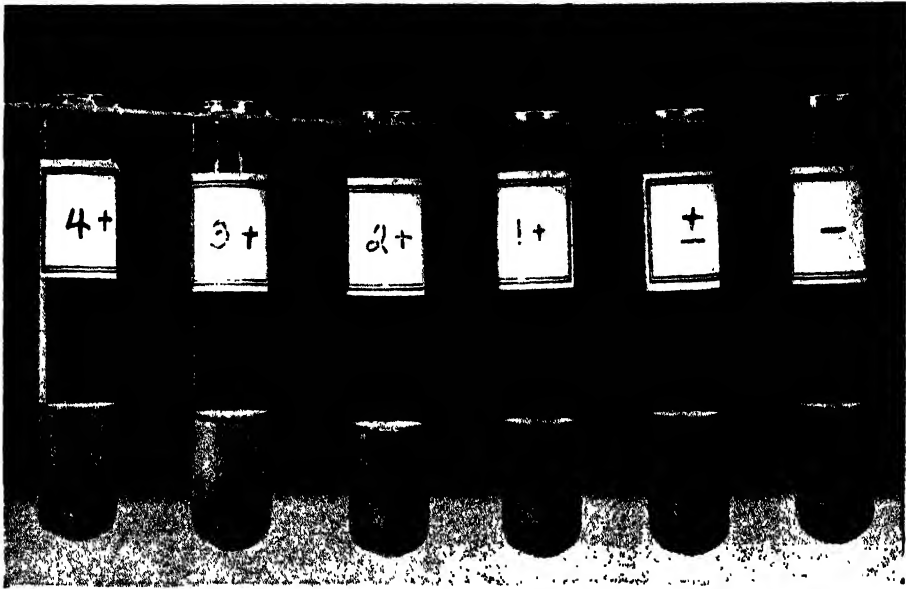
8. The secondary incubation is conducted and the reactions read as described above for the regular methods.

**Reading Scales.**—Until considerable experience has been gained in the reading of reactions, it is advisable to prepare scales showing the appearance of + + + +, + + +, + +, +,  $\pm$  and — complement fixation reactions according to the method employed (Plate XV). A + reaction indicates about 10 per cent inhibition of hemolysis while a  $\pm$  reaction is less than 10 per cent. By carrying over in the refrigerator a 2 per cent suspension of washed sheep corpuscles and 4 tubes showing complete hemolysis in the hemolysin titrations, designated as hemoglobin solutions, they may be prepared with sufficient accuracy during the secondary incubation of the tests as shown in Table 45.

**Reactions and Interpretations.**—All serum, spinal fluid, antigen and hemolytic system controls should show complete hemolysis. The corpuscle controls should show no hemolysis. The reactions should be recorded for each tube of complement fixation tests as: + + + + (4), + + + (3), + + (2), + (1),  $\pm$  or —.

1. The reactions in *quantitative or six-tube tests* with serum or spinal fluid may be interpreted as follows: (a) *Very strongly positive* when complete fixation (+ + + +) occurs in any of the first 3, 4 or 5 tubes, like 3 4 4 — —, 4 4 4 — —, 4 4 4 2 — —, 4 4 4 3 — — or 4 4 4 4 4; (b) *strongly positive* when complete fixation (+ + + +) occurs in the second tube like 4 4 3 1 — —, 4 4 2 — —, 3 4 2 — — or 4 4 — — —; (c) *moderately positive* when complete fixation (+ + + +) occurs in the first tube only like 4 3 1 — —, 4 2 — — — or 4 — — — —; (d) *weakly positive* when partial fixation occurs in one or more tubes like 3 2 1 — —, 2 1 — — — or 1 — — — —; (e) *doubtful* when the reaction is  $\pm$  in the first tube like  $\pm$  — — — — and (f) *negative* when there is complete hemolysis in all tubes (— — — — —).

PLATE XV



READING SCALE FOR RECORDING KOLMER COMPLEMENT FIXATION REACTIONS



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TABLE 45.—PREPARATION OF READING SCALES

Tests	Corpuscle Suspensions * cc.	Hemoglobin Solutions ** cc.	Equivalent in Complement Fixation
Regular	3.0	—	100% (++++ or 4)
	1.5	1.5	50% (+++ or 3)
	0.75	2.25	25% (++ or 2)
	0.3	2.7	10% (+ or 1)
	0.15	2.85	5% (±)
	—	3.0	0 (—)
One-Half	1.5	0	100% (++++ or 4)
	0.75	0.75	50% (+++ or 3)
	0.37	1.13	25% (++ or 2)
	0.15	1.35	10% (+ or 1)
	0.07	1.43	5% (±)
	—	1.5	0 (—)
One-Fifth	0.7	—	100% (++++ or 4)
	0.35	0.35	50% (+++ or 3)
	0.18	0.52	25% (++ or 2)
	0.07	0.63	10% (+ or 1)
	0.03	0.67	5% (±)
	—	0.7	0 (—)

\* 1:6 dilution of 2 per cent suspension for regular method; 1:5 dilution of 2 per cent suspension for one-half method; 1:7 dilution of 2 per cent suspension for one-fifth method.

\*\* Employing tubes showing complete hemolysis in the hemolysin titrations of the regular, one-half and one-fifth methods respectively.

Otherwise the results may be reported in terms of *Kolmer units* in which the potency of any serum or spinal fluid is determined according to the formula  $S = 4D$  as used in the Kahn quantitative serum test, where *S* is the serum or spinal fluid potency in terms of units and *D* is the highest dilution giving a positive (++++, ++++, ++ or +) reaction.

If a *serum* gives a + + + +, + + +, ++ or + reaction in the first tube only and is negative in the remaining 4 tubes, it is considered as containing Kolmer units as indicated by the plus signs (4 units, 3 units, 2 units or 1 unit, respectively). If a reaction of any degree occurs in the second tube ( $D = 2$ ) and negative in the remaining 3 tubes, it is considered as containing 8 Kolmer units; if a reaction of any degree occurs in the third tube ( $D = 4$ ) and is negative in the remaining 2 tubes, it is considered as containing 16 Kolmer units; if a reaction of any degree occurs in the fourth tube ( $D = 8$ ) and is negative in the fifth, it is considered as containing 32 Kolmer units; if a reaction of any degree occurs in the fifth tube ( $D = 40$ ) it is considered as containing 160 or more Kolmer units. In this case still higher dilutions of serum may be tested if desired.

If a *spinal fluid* gives a + + + +, + + +, ++, or + reaction in the first tube only and is negative in the remaining 4 tubes, it is considered as containing Kolmer units as indicated by the plus signs (4 units, 3 units, 2 units, or 1 unit, respectively).

If a reaction of any degree occurs in the second tube ( $D = 2$ ) and is negative in the remaining 3 tubes, it is considered as containing 8 Kolmer units; if a reaction of any degree occurs in the third tube ( $D = 4$ ) and is negative in the remaining 2 tubes, it is considered as containing 16 Kolmer units; if a reaction of any degree occurs in the fourth tube ( $D = 8$ ) and is negative in the fifth, it is considered as containing 32 units; if a reaction of any degree occurs in the fifth tube ( $D = 16$ ), it is considered as containing 64 or more Kolmer units. In this case still higher dilutions of spinal fluid may be tested if desired.

*Weakly anticomplementary reactions* may be safely reported as follows 4 4 4 1 — 2 = positive; 4 4 1 — — 1 = positive; 4 4 1 — — ± = positive; 3 2 — — — ± = positive; 4 4 1 — — 3 = doubtful; 3 2 — — — 1 = doubtful; 2 1 — — — ± = doubtful; 3 — — — — ± = doubtful; 1 — — — — ± = negative; 1 — — — — 1 = negative; 2 — — — — 1 = negative; 2 — — — — 2 = negative.

2. The reactions in the *simplified test using three tubes* may be interpreted as follows: (a) *Strongly positive* when complete fixation (· · + + +) occurs in the second tube like 4 4 or 3 4; (b) *moderately positive* when complete fixation (+ + + +) occurs in the first tube only, like 4 2 or 4 1; (c) *weakly positive* when partial fixation occurs in one or both tubes, like 3 1, 2 1, 3 —, 2 — or 1 —; (d) *doubtful* when the reaction is ± in the first tube, like ± — and (e) *negative* when there is complete hemolysis in both tubes (— —).

3. The reactions in the *simplified test using two tubes* may be interpreted as *strongly positive* (+ + + +); *moderately positive* (+ + +); *weakly positive* (+ + or +); *doubtful* (±) or *negative* (—).

Slightly *anticomplementary reactions* may be safely reported as follows: 4 ± positive; 4 1 = positive; 4 2 = doubtful; 3 1 = doubtful; 3 ± = doubtful; 2 2 = negative; 2 1 = negative; 1 1 = negative; 1 ± = negative; 1 ± = negative; ± ± = negative.

4. But until considerable experience has been gained it is always advisable to repeat the tests with fresh sera when anticomplementary reactions occur and especially in the case of doubtful reactions. Sera which are heavily contaminated with bacteria and those which are chylous or heavily discolored with hemoglobin or bilirubin and thereby strongly anticomplementary, may be prepared by a modified Sachs method, described below.

5. The Committee on the Evaluation of Serodiagnostic Tests for Syphilis of the U. S. Public Health Service recommends reporting all reactions merely as positive, doubtful or negative.

**Analysis of Difficulties.**—*Due to Complement.*—When difficulties are experienced they are usually first ascribed to defective hemolysin or antigen but, since both of these keep very well, they are rarely responsible. In some instances difficulties are due to the use of complement too low in hemolytic activity and particularly in the case of preserved complement. This is especially likely to be the case during the hot months of the year and likewise when the complement sera of underweight or pregnant guinea-pigs are used, as well as those previously employed in various inoculation tests. Under these circumstances anticomplementary reactions may occur with incomplete hemolysis of the antigen, serum and spinal fluid controls. When the unit of complement is more than 0.5 cc. of 1:30 dilution in the regular tests it should not be employed.

Furthermore, while the complement may be satisfactory from the standpoint of hemolytic activity, it may be defective because it is supersensitive to what may be called the "occult" anticomplementary effects of antigen, serum or spinal fluid. Under these circumstances inhibition of hemolysis occurs in those tubes carrying antigen while the antigen, serum and spinal fluid controls show complete hemolysis with the danger of reporting falsely positive or nonspecific reactions. When pretested complement or the pooled complement of a large number of guinea-pigs is employed they are of exceptional occurrence. However, since they may occur in quantitative and simplified spinal fluid tests, it is recommended that egg albumin be used routinely in their conduct, especially if pretested complement is not being employed.

*Due to Prezone Reactions.*—Prezone reactions may occur in quantitative tests with sera giving reactions like — — 234, with complete hemolysis of the serum control, but sometimes with incomplete hemolysis of the antigen control. They occur quite infrequently when pretested complement or the pooled complement of a large number of guinea-pigs is employed. Otherwise, they may be prevented by using egg albumin as previously described.

*Due to Hemolysin.*—As previously stated, this is usually first suspected but is usually least likely to be a cause of difficulty and especially if the hemolysin has been previously found satisfactory. The unit of antsheep hemolysin in the regular tests should be at least 0.5 cc. of 1:4000 and hemolysins of this and higher strengths are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible even when shipped over long distances or kept in a refrigerator over months and even years of time.

*Due to Corpuscles.*—When blood is obtained from an abattoir one is almost sure, sooner or later, to encounter the corpuscles of occasional animals possessing increased resistance to serum hemolysis. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the corpuscles and secure a fresh supply of blood. When the corpuscles of preserved blood tend to become too fragile, it is advisable to use 0.9 instead of 0.85 per cent saline solution in the conduct of the tests.

*Due to Antigen.*—Providing no mistakes have occurred in dilution and dosage, this is very rarely a cause of trouble. When the antigen control shows incomplete hemolysis it is almost surely due to some component of the hemolytic system, especially complement supersensitive to antigen, in which case egg albumin may be employed.

*Due to Anticomplementary Sera and Spinal Fluids.*—Sera and spinal fluids may be found to be anticomplementary, as shown by incomplete hemolysis of the controls. After experience has been gained some of these reactions may be safely read, as previously stated, but as a general rule it is safer and wiser to repeat the tests with fresh serum or spinal fluid, especially in the case of those technicians lacking experience in complement fixation work. It is infinitely better to repeat the tests than to run the slightest chance of error, especially the regrettable error of rendering a falsely positive report. Sometimes the majority of sera and spinal fluids of a day's work show incomplete hemolysis of the controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests may have to be repeated and for this reason the *unused portions of all sera and spinal fluids should be routinely kept in a refrigerator until the tests are completed in case repetitions are required.* In case difficulties are due to the presence of thermostable anticomplementary substances in sera they can usually be

satisfactorily tested after preparation by the modified method of Sachs including the use of egg albumin.

**Methods for Testing Anticomplementary Sera.**—As previously stated, the results of tests with weakly anticomplementary sera may be sometimes safely reported in terms of positive, doubtful or negative. When strongly anticomplementary, they may be prepared according to a modified Sachs method as follows:

1. Heat 0.5 cc. at 55° to 56° C. in a water bath for 15 minutes. If the serum has been previously heated and is being retested, heat for 10 minutes.

2. Add 4.1 cc. of accurately titrated N/300 hydrochloric acid and mix thoroughly.

3. After standing  $\frac{1}{2}$  hour at room temperature, centrifuge thoroughly and discard the sediment.

4. To the supernatant fluid add 0.4 cc. of 10 per cent sodium chloride solution. The acid is fixed by the precipitate of globulin; hence neutralization is unnecessary.

5. This gives a 1:10 dilution of original serum ready for testing as follows:

**Regular Quantitative Test.**—1. Arrange 2 rows of 5 test tubes (the rear tubes are serum controls).

2. Place 1 cc. of normal saline solution in tubes 3 and 4 and 2 cc. in tube 5 of the first row; place 0.5 cc. in each of the 5 tubes of the second row (serum controls).

3. Place 1 cc. of prepared serum (1:10) in the first and third tubes of the first row and 0.5 cc. in the second tube. Mix No. 3 and transfer 1 cc. to No. 4 and 0.5 cc. to No. 3 of the second row. Mix No. 4; transfer 0.5 cc. to No. 5, 0.5 cc. to No. 4 of the second row and discard 0.5 cc. Mix No. 5, transfer 0.5 cc. to No. 5 of the rear row and discard 1.5 cc.

4. Place 1 cc. of prepared serum 1:10 in No. 1 and 0.5 cc. in No. 2 of the second row.

5. The 5 tubes of each row now carry 0.1, 0.05, 0.025, 0.0125 and 0.0025 cc. of serum, respectively.

6. Add antigen (0.5 cc. of proper dilution) to each tube of the front row.

7. Mix the contents of all tubes and allow to stand at room temperature for 10 to 30 minutes. If a longer time elapses place the tubes in a refrigerator.

8. Add 1 cc. of complement (2 full units) and 0.2 cc. of 50 per cent egg albumin to each of the 5 tubes in both rows. Also add 0.2 cc. of 50 per cent egg albumin to the antigen control. Or dilute the complement with a 10 per cent solution of egg albumin in saline solution instead of with plain saline (1 cc. to carry 2 full units) and add 1 cc. to all tubes of both rows including the antigen control. Complete the test in the usual manner.

9. Upon completion of the test all of the tubes of the rear row should show complete hemolysis. However, the first tubes carrying 0.1 cc. and sometimes the second tubes carrying 0.05 cc. of serum of both rows, may show slight inhibition of hemolysis. With negative sera the corresponding front tubes show the same inhibition of hemolysis and if the degree of inhibition is slight, a negative report may be rendered. With positive sera inhibition of hemolysis is much more marked in the tubes of the front row. It is advisable to report the reactions as positive, doubtful, or negative.

**Regular Simplified Test.**—1. Arrange 2 rows of 3 test tubes each (the rear tubes are serum controls).

2. Place 0.5 cc. of normal saline solution in the third tube of the first row and 0.5 cc. in each of the 3 tubes of the rear row (serum controls).

3. Place 1 cc. of prepared serum (1:10) in the first tube of the front row and 1 cc. in the first tube of the second row.

4. Place 0.5 cc. of prepared serum (1:10) in the second tube of the front row and 0.5 cc. in the second tube of the rear row.

5. Place 0.5 cc. of prepared serum (1:10) in the third tube of the first row; mix and transfer 0.5 cc. to the third tube of the rear row.

6. The 3 tubes of each row now carry 0.1, 0.05 and 0.025 cc. of serum respectively.

7. Add antigen (0.5 cc. of proper dilution) to each tube of the front row.

8. Mix the contents of all tubes and allow to stand at room temperature for 10 to 30 minutes. If a longer time elapses place the tubes in a refrigerator.

9. Add 1 cc. of complement (2 full units) and 0.2 cc. of 50 per cent egg albumin to each of the 3 tubes in both rows. Also add 0.2 cc. of 50 per cent egg albumin to the antigen control. Or dilute the complement with a 10 per cent solution of egg albumin in saline solution instead of with plain saline (1 cc. to carry 2 full units) and add 1 cc. to all tubes of both rows including the antigen control. Complete the test in the usual manner.

10. Upon completion of the test all of the tubes of the rear row should show complete hemolysis. However, the first tubes carrying 0.1 cc. and sometimes the second tubes carrying 0.05 cc. of serum, of both rows, may show slight inhibition of hemolysis. With negative sera the corresponding front tubes show the same degree of inhibition of hemolysis, and if the degree of inhibition is slight, a negative report may be rendered. With positive sera inhibition of hemolysis is much more marked in the tubes of the front row. It is advisable to report the reactions as positive, doubtful or negative.

*One-Fifth Quantitative Test.*—1. Arrange 2 rows of 5 test tubes (the rear tubes are serum controls).

2. Place 0.2 cc. of saline solution in Nos. 2, 3 and 4 of both rows and 0.8 cc. in No. 5 of each row.

3. Place 0.2 cc. of prepared serum 1:10 in Nos. 1 and 2 of the front row. Mix No. 2 and transfer 0.2 cc. to No. 3. Mix No. 3 and transfer 0.2 cc. to No. 4. Mix No. 4 and transfer 0.2 cc. to No. 5. Mix No. 5 and discard 0.8 cc.

4. Repeat in the same manner with the rear row.

5. The 5 tubes of each row now carry 0.02, 0.01, 0.005, 0.0025 and 0.0005 cc. of serum respectively.

6. Add antigen (0.1 cc. of proper dilution) to each tube of the front row.

7. Mix the contents of all tubes and allow to stand at room temperature for 10 to 30 minutes. If a longer period elapses place the tubes in a refrigerator.

8. Add 0.2 cc. complement (2 full units) and 0.2 cc. of 10 per cent egg albumin to each of the 5 tubes of both rows. Also add 0.2 cc. of 10 per cent egg albumin to the antigen control. Or dilute the complement with a 10 per cent solution of egg albumin in saline solution instead of with plain saline (0.2 cc. to carry 2 full units) and add 0.2 cc. to all tubes of both rows including the antigen control. Complete the test in the usual manner.

Upon completion of the test all of the tubes in the rear row should show complete hemolysis. However, the first tubes carrying 0.02 cc. and sometimes the second tubes carrying 0.01 cc. of serum, of both rows, may show a slight inhibition of hemolysis. With negative sera the corresponding front tubes show the same degree of inhibition of

hemolysis and if the degree of inhibition is slight, a negative report may be rendered. With positive sera inhibition of hemolysis is much more marked in the tubes of the front row. It is advisable to report the reactions as positive, doubtful or negative.

**Method for Testing Anticomplementary Spinal Fluids.**—As previously stated, the results of tests with weakly anticomplementary spinal fluids may be sometimes safely reported in terms of positive, doubtful or negative. Strongly anticomplementary fluids may be tested according to the technic of the regular, one-half or one-fifth *quantitative* methods by setting up 2 rows of 5 tubes each. Antigen is added to each tube of the first row but is omitted from those in the second row, which serve as controls on each dose of fluid in the front row. The tests are then completed in the usual manner and the reactions interpreted as per the following examples:

First row:	4	4	4	1	—	} Positive
Second row:	4	1	—	—	—	

First row:	4	3	2	—	—	} Positive
Second row:	4	1	—	—	—	

First row:	4	1	—	—	—	} Positive
Second row:	1	—	—	—	—	

First row:	4	4	2	—	—	} Negative
Second row:	4	2	1	—	—	

First row:	4	2	—	—	—	} Negative
Second row:	4	1	—	—	—	

First row:	3	2	—	—	—	} Negative
Second row:	3	1	—	—	—	

### KOLMER COMPLEMENT FIXATION TESTS FOR BACTERIAL, SPIROCHAETAL AND VIRAL DISEASES

The technic is exactly the same as in the Kolmer complement fixation test for syphilis except in the methods employed for the preparation and titration of antigens. As in agglutination tests, certain strains of micro-organisms may be more suitable than others for the preparation of antigens. Consequently, the methods employed for the preparation and titration of antigens have a marked influence upon the sensitivity and specificity of complement fixation reactions in the bacterial diseases.

**Gonococcus Antigen.**—This antigen may be prepared of 10 to 14 freshly isolated strains, or five or more of the so-called "Torrey strains" of gonococci cultivated on a suitable solid medium with the hope of covering the range of serological variation in the group. After 24 to 48 hours' incubation the growths are removed with saline solution, diluted to contain about 3000 million per cc., heated in a water bath for an hour at 60° C. and preserved with 0.5 per cent phenol. This method gives a good yield but the antigen may be lacking in acceptable sensitivity and specificity.

In the *method of Price as modified by Torrey (Jour. Immunol. 38: 413, 1940)* the antigen is prepared of the "Uri" and "J.G." strains of gonococci cultivated in Kolle culture flasks of "hormone" veal peptone agar seeded with suspensions from 24-hour growths on slants of the same medium harvested with 1:5 sterile ascites fluid in saline solution. After 24-hour incubation at 36° C. the growth from each flask is suspended in

100 cc. of normal saline solution. After adding 1 cc. of normal sodium hydroxide solution to each 100 cc., the suspension is placed in a water bath at 37° C. for 2 hours followed by filtration through sterile lint. To the filtrate is added sufficient 10 per cent trichloroacetic acid to start precipitation. The amount required varies from 1.5 to 2.5 cc. per 100 cc., precipitation generally occurring between pH 6.4 and 6.6. After 20 minutes at 37° C., the flocculent precipitate is collected by centrifuging and the precipitate from each 100 cc. is suspended in 4 cc. of saline solution. Tenth normal NaOH is then gradually added to a pH of 7.5 when the fluid becomes clear, or nearly so. Merthiolate to a 1:10,000 concentration is added as a preservative.

Antigen prepared according to the *method of Cohn (Jour. Lab. and Clin. Med. 22: 627, 1936-37)* is a simple autolysate of gonococcal strains and requires about 3 months to ripen. It may be prepared by washing off growths in each Kolle flask with 10 cc. normal saline solution. To each 90 cc. of mixed suspension is added 10 cc. of 5 per cent phenol followed by storage in the refrigerator for 3 months with occasional shaking.

According to the *method of McNeil (Proc. Soc. Exper. Biol. and Med. 29: 983, 1931-32)* 18- to 24-hour cultures of gonococci are harvested with saline solution and the suspensions thoroughly centrifuged. The sediment of gonococci is then treated with alcohol followed by ether and dried. One gram of dried powder is suspended in 200 cc. of freshly doubled distilled water, placed in a water bath at 55° C. for 30 minutes with frequent shaking and then centrifuged at high speed for 30 minutes. The supernatant fluid is discarded and the remaining protein residue suspended in 200 cc. of 1:10,000 merthiolate in 0.9 per cent saline solution.

**Tuberculosis Antigen.**—1. Cultivate human tubercle bacilli in glycerin broth for about 4 weeks and autoclave the flasks at 10 pounds pressure for 20 minutes to kill the organisms.

2. Filter on several layers of good paper and wash the bacillary residue free of glycerin with sterile water.

3. Transfer the residue to a desiccator and dry over sulphuric acid.

4. Grind in a mortar under a hood for ½ hour and keep in a tightly stoppered bottle at room temperature.

5. Place 1 gram of powder in a small Erlenmeyer flask fitted with a Liebig's condenser and electric heater and boil gently for 1 hour with 200 cc. of ether. Discard the ether; dry the residue by placing the flask in an incubator, add 200 cc. of acetone and boil for 1 hour. Discard the acetone, add 200 cc. of absolute ethyl alcohol and boil for 1 hour. Discard the alcohol.

6. Dry the residue in the flask, add 190 cc. of distilled water and boil for 1 hour. Add 2 grams sodium chloride to render isotonic and 10 cc. of 5 per cent tricresol or phenol as a preservative. Store in a tightly stoppered bottle in a refrigerator for at least 1 week to ripen before titration.

**Typhoid and Glanders Antigens.**—1. Cultivate the micro-organisms on a suitable solid medium and wash off the growths with sufficient sterile distilled water to give a suspension containing approximately 2,000,000,000 per cc. Or the organism may be cultivated in a suitable fluid medium, centrifuged and the residue suspended in sterile water to the same concentration.

2. Shake the suspension with glass beads for an hour to break up clumps.

3. Transfer to an Erlenmeyer flask fitted with a Liebig's condenser and electric



heater and boil gently for 2 hours. Or the suspension may be boiled in an Arnold sterilizer for the same time, making up for any loss in volume with sterile water.

4. Add 1 gram of sodium chloride and 5 cc. of 5 per cent tricresol or phenol to each 100 cc.; stopper tightly and keep in a refrigerator for a week to ripen before titrating.

The following method is particularly serviceable for preparing antigens of organisms readily secured in large amounts, as storing in dry powdered form provides a means of keeping indefinitely the base from which antigen may be made up in small amounts as required.

1. Cultivate the organism on a suitable solid medium and remove with a *minimum* amount of sterile saline solution or cultivate in a fluid medium and secure the organisms by centrifugation.

2. Dry the residue in a desiccator over sulphuric acid and grind for  $\frac{1}{2}$  hour under a hood.

3. Store the powder in ampules or in a tightly stoppered bottle at room temperature.

4. For use place 1 gram in 190 cc. of sterile distilled water and boil with a condenser for 2 hours; or boil in an Arnold sterilizer for the same time, making up for any loss in volume by adding distilled water.

5. Add 2 grams of sodium chloride and 10 cc. of 5 per cent phenol or tricresol. Place in a tightly stoppered bottle in a refrigerator to ripen for a week before titrating.

In case one so desires smaller quantities can readily be made up in similar proportions.

**Brucella Abortus Antigen.**—According to the *method of Boerner and Stubbs*, cultivate several strains on liver infusion or glycerin agar for 4 to 7 days. Wash off with small amounts of sterile distilled water. Heat at 100° C. for 3 hours and place in refrigerator for 10 days with occasional shakings. Then shake well and centrifuge at low speed for a short time to throw down a greater portion of the bacteria. The supernatant fluid, which should be still quite turbid, is pipetted off; add phenol to 0.5 per cent. Keep in refrigerator.

**Salmonella Pullorum Antigen.**—According to the *method of Bushnell and Hudson* cultivate *Salmonella pullorum* on agar. Wash off with saline solution. Shake vigorously for several minutes and filter through glass wool. Centrifuge for 45 to 60 minutes at high speed. Discard the supernatant fluid. To each cc. of sediment add 10 cc. of ether. Mix well for 4 hours. Discard the ether. Add fresh ether and extract for 2 hours. Discard ether and dry residue in incubator. Suspend the residue in sufficient saline solution to give a turbidity equal to tube 3 of the McFarland nephelometer.

**Spirochaetal Antigen.**—Spirochaetal antigens for the complement fixation test in syphilis may be prepared, according to the *method of Kast and Kolmer*, of pure cultures of the Reiter or other strains of *Treponema pallidum* by cultivation in heart infusion broth (Difco) containing 0.05 per cent thioglycollate and 10 per cent sterile horse serum for 5 to 7 days in 125 cc. round-bottomed flasks with vaseline seals. The sterile horse serum should be heated beforehand in a water bath at 63° to 65° C. for 30 to 60 minutes and thoroughly centrifuged for the removal of any precipitate. The culture is then centrifuged for 1½ hours at approximately 3000 r.p.m. in a conical head International centrifuge and the sediment of spirochetes washed twice with large volumes of sterile saline solution. After the last washing the spirochetes are

suspended in sufficient 0.5 per cent solution of phenol in saline solution to give a turbidity approximately equal to a reading of 450 on the Kleet-Summerson colorimeter. Upon completion the antigen is kept in a refrigerator and thoroughly shaken before titration and use in the complement fixation test.

**Lymphopathia Venereum Antigen.**—This antigen is essentially a saline suspension rich in virus elementary bodies, derived from the yolk sacs of embryos moribund following infection via this route, prepared according to the *method of Shaffer, Rake and Grace (Am. Jour. Syph., Gonorr. and Ven. Dis. 26: 271, 1942)*. The preparation is freed from the bulk of tissue constituents by differential centrifugation and treated with formalin, urea or ether to inactivate the virus while usually maintaining its antigenicity.

**Antigens for Virus Infections of the Central Nervous System.**—Satisfactory antigens may be prepared according to the *method of Casals (Jour. Exper. Med. 74: 409, 1941; Proc. Soc. Exper. Biol. and Med. 49: 501, 1942)* for complement fixation tests for rabies, St. Louis encephalitis, Japanese B encephalitis, lymphocytic choriomeningitis, Eastern and Western equine encephalomyelitis and louping ill by obtaining infected mouse brains, making them into a 10 per cent suspension with diluent in a mechanical homogenizer, centrifugalizing the material at 2500 r.p.m. in a horizontal centrifuge, freezing and thawing it, and finally centrifuging it at 5000 r.p.m. in an angle-head centrifuge and discarding the sediment. These preparations are then rendered non-virulent by exposure to the rays of a mercury arc lamp for a determined period of time.

Freezing and drying of these antigens are accomplished in the following manner. The irradiated antigen is freed of sediment by centrifugation in a horizontal centrifuge for 10 minutes at 2500 r.p.m. To the clear supernatant, merthiolate in a dilution of 1:10,000 is added. The antigen is then pipetted in 2 or 5 cc. quantities into glass ampules, frozen quickly by immersion into a dry ice-alcohol mixture, and dried over a period of 20 hours in a Flosdorf-Mudd apparatus, after which the ampules are sealed. The ampules containing the desiccated antigen in an air-free space are stored at 2° C. When needed for use in tests the ampules are opened and 2 or 5 cc. of distilled water added to the desiccated material.

**Anticomplementary Titrations.**—This is for the purpose of determining the *anticomplementary unit which is the smallest amount of antigen giving slight inhibition of hemolysis at the end of the secondary incubation of one hour in a water bath at 37 C.* Dilutions of antigen are prepared with saline solution and it makes no difference whether antigen is added to saline or saline to antigen. The method of titration is shown in Table 46. The hemolytic system control should show complete hemolysis; the corpuscle control should show no hemolysis.

**Antigenic Titration.**—This titration is advisable (but not absolutely essential) if a known positive serum is available. A mixture of positive *human* sera should be employed (after heating in a water bath at 55° to 60° C. for 30 minutes) in dose of 0.5 cc. of 1:10 dilution. The same applies to *horse* sera and *guinea-pig* immune sera. *Cattle* sera should be heated at 58° to 60° C. and *mule* sera at 62° C. for 30 minutes and used in dose of 0.5 cc. of 1:10 dilution. Immune *rabbit* sera should be heated to 60° to 62° C. for 30 minutes and used in dose of 0.5 cc. of 1:50 or higher in order to avoid nonspecific reactions. The *antigenic unit is the smallest amount of antigen giving a ++++ reaction*. The method is shown in Table 47. The serum and hemo-

TABLE 46.—ANTICOMPLEMENTARY TITRATION

Tube	Antigen 0.5 cc.	Saline Solution cc.	Complement cc. (2 full units)		Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)	
1	Undiluted	0.5	1.0	Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 30 minutes in water bath at 37° C.	0.5	0.5	Mix all tubes. Secondary incubation in water bath at 37° C. for 1 hour; make reading.
2	1:2	0.5	1.0		0.5	0.5	
3	1:3	0.5	1.0		0.5	0.5	
4	1:4	0.5	1.0		0.5	0.5	
5	1:6	0.5	1.0		0.5	0.5	
6	1:8	0.5	1.0		0.5	0.5	
7	1:10	0.5	1.0		0.5	0.5	
8	1:12	0.5	1.0		0.5	0.5	
9	1:16	0.5	1.0		0.5	0.5	
10	1:20	0.5	1.0		0.5	0.5	
11	Hemolytic Control	1.0	1.0		0.5	0.5	
12	Corpuscle Control	2.5	—		—	0.5	

lytic system controls should show complete hemolysis; the corpuscle control should show no hemolysis.

**Amount of Antigen to Employ.**—An antigen may be used in an amount equivalent to about  $\frac{1}{3}$  or  $\frac{1}{4}$  of its anticomplementary unit in the conduct of the complement fixation test. For example, if the anticomplementary unit is 0.5 cc. of 1:6 dilution, the dose may be 0.5 cc. of 1:18 to 1:24 dilution.

If an antigenic titration has been conducted the dose should be equivalent to at least 2 to 10 antigenic units providing this amount is no more than  $\frac{1}{3}$  the anticomplementary unit.

Bacterial antigens usually keep quite well in a refrigerator but it is advisable to retitrate them every 2 or 3 months for possible increase of anticomplementary activity or loss of antigenic sensitivity.

**Quantitative and Simplified Complement Fixation Tests.**—The technic of these is exactly as described for the syphilis tests except that the primary incubation may be either (a) 15 to 18 hours in the refrigerator at 6° to 8° C. followed by  $\frac{1}{2}$  hour in a water bath at 37° C. or (b) 2 hours in a water bath. The latter is particularly recommended for the tuberculosis complement fixation test. In the *quantitative* test serum is used in amounts of 0.2, 0.1, 0.05, 0.25 and 0.005 cc. with 0.2 cc. in the

TABLE 47.—ANTIGENIC TITRATION

Tube	Antigen 0.5 cc.	Positive Serum cc.	Complement cc. (2 full units)	Mix all tubes. Primary incubation 6°-8° C. for 15-18 hours followed by 30 minutes in water bath at 37° C.	Hemolysin cc. (2 units)	Corpuscles cc. (2 per cent)	Mix all tubes. Secondary incubation in water bath at 37° C. for 1 hour; make reading.
1	1:10	0.5	1.0		0.5	0.5	
2	1:20	0.5	1.0		0.5	0.5	
3	1:40	0.5	1.0		0.5	0.5	
4	1:60	0.5	1.0		0.5	0.5	
5	1:80	0.5	1.0		0.5	0.5	
6	1:100	0.5	1.0		0.5	0.5	
7	1:200	0.5	1.0		0.5	0.5	
8	1:300	0.5	1.0		0.5	0.5	
9	1:400	0.5	1.0		0.5	0.5	
10	Serum control 0.5 cc. saline	0.5	1.0		0.5	0.5	
11	Hemo. control 1.0 cc. saline	--	1.0		0.5	0.5	
12	Corp. control 2.5 cc. saline	--	—		—	0.5	

control; in the *simplified test* the serum is used in amounts of 0.2 and 0.1 cc. with 0.2 cc. in the control.

It is advisable to include positive and negative controls, especially the former. The negative controls should be of human sera in the tuberculosis, gonococcus, typhoid and such tests; the positive controls should be of human sera when available but otherwise immune sera of the lower animals may be used.

*The readings should be made 10 minutes after complete hemolysis of the antigen control in the case of those sera showing complete hemolysis of the serum controls; these give the most sensitive readings. Otherwise the readings should be made immediately after the secondary incubation of 1 hour, providing the antigen, serum, hemolytic system and negative serum controls show complete hemolysis.*

*In conducting the tuberculosis complement fixation test, the Wassermann test should be always conducted at the same time because syphilis antibody may give a positive reaction with tuberculosis antigen in the absence of tuberculosis. When the Wassermann reaction is strongly positive, the tuberculosis test is also quite apt to yield a positive reaction and should be reported upon with great caution. This is not true,*

however, in the case of the gonococcus, typhoid and other bacterial complement fixation tests.

### KOLMER COMPLEMENT FIXATION TESTS WITH RABBIT, DOG AND MULE SERA

The sera of some of the lower animals, notably of the rabbit, dog, and mule, sometimes yield nonspecific complement fixation and precipitation reactions with the various antigens employed in the syphilis reactions. With various bacterial antigens the degree of fixation is even greater. *Therefore, in conducting complement fixation reactions with the sera of these animals, the technic must be modified to avoid the possibility of these nonspecific reactions and yet sufficiently sensitive for the detection of specific antibody.* These ends are met by heating the sera at 62° C. instead of at 55° to 56° C. and by using more complement (first method) or smaller doses of serum (second method).

**First Method.**—*The technic is exactly the same as described for the testing of human sera except:*

1. The natural antisheep hemolysins are not removed from the sera.
2. The sera are heated in a water bath at 62° C. for 30 minutes.
3. The doses in *syphilis* tests are 0.1, 0.05, 0.025, 0.0125, 0.006 and 0.1 cc. (control) in the quantitative test. But in *bacterial* complement fixation tests smaller amounts of serum should be used as:

0.025 cc. (0.5 cc. of 1:20)  
0.012 cc. (0.5 cc. of 1:40)  
0.006 cc. (0.5 cc. of 1:80)  
0.003 cc. (0.5 cc. of 1:160), etc.  
0.025 cc. (0.5 cc. of 1:20) control

The first 2 doses of either series are used in the qualitative test, *i.e.*, 0.1, 0.05 and 0.1 cc. (control) with cholesterolized and lecithinized alcoholic extracts of beef heart antigen in syphilis test, or 0.025, 0.012 and 0.025 cc. (control) in bacterial complement fixation tests employing the antigen in a dose equivalent to *one-fourth* of its anticomplementary unit.

4. *Four units of complement* are used instead of 2½ and so diluted that this dose is contained in 1 cc. Example:

Unit = 0.3 cc. of 1:30  
Four units = 1.2 cc. of 1:30

To calculate the dilution to use so that 1 cc. contains the dose, divide 30 by the dose:

$$\frac{30}{1.2} = 25 \text{ or dilution } 1:25 \text{ in dose of } 1 \text{ cc.}$$

**Second Method.**—*The technic is exactly the same as described for testing human sera except:*

1. The natural antisheep hemolysins are not removed from the sera.
2. The sera are heated in a water bath at 62° C. for 30 minutes.

3. The doses of serum in quantitative *syphilis* tests with the usual dose of antigen are:

0.025 cc. (0.5 cc. of 1:20)  
0.0125 cc. (0.5 cc. of 1:40)  
0.006 cc. (0.5 cc. of 1:80)  
0.003 cc. (0.5 cc. of 1:160)  
0.0015 cc. (0.5 cc. of 1:320)  
0.025 cc. (0.5 cc. of 1:20) control

The doses in quantitative *bacterial* tests with one-fourth of the anticomplementary unit of antigen are:

0.0125 cc. (0.5 cc. of 1:40)  
0.006 cc. (0.5 cc. of 1:80)  
0.003 cc. (0.5 cc. of 1:160)  
0.0015 cc. (0.5 cc. of 1:320)  
0.0008 cc. (0.5 cc. of 1:640)  
0.0125 cc. (0.5 cc. of 1:40) control

In qualitative tests the first 2 doses of either series are employed along with the larger amount in the third tube or serum control.

*Heating sera at 62° C. for 30 minutes does not destroy enough antibody in syphilitic rabbits or in rabbits and dogs immunized to various antigens to interfere with the sensitiveness of the reactions; nor does this degree of heating of mule sera interfere with the sensitiveness of the glanders complement fixation test for which they are usually submitted.*

### KOLMER COMPLEMENT FIXATION TESTS WITH URINE, MILK, TRANSUDATES AND EXUDATES

Transudates like pleural, pericardial, peritoneal and joint fluids are usually free of anticomplementary activity and may be tested in the same manner as serum. They should be heated at 55° C. for 15 minutes. As a general rule, however, their antibody content is less than in serum and larger doses are sometimes required similar to those employed in testing spinal fluid (0.5, 0.25, 0.125 cc., etc.).

Exudates like blister fluids and tuberculous pleural exudates are much more likely to be anticomplementary; likewise urine and milk.

*Urine, milk and exudates* (like pleural exudates for tuberculosis) should be freshly collected and kept at a low temperature until examined. Each should be heated at 55° C. for 15 minutes and first tested for anticomplementary activity as follows:

1. Titrate hemolysin.
2. In a series of eight test tubes place 0.5 cc. undiluted, 1:2, 1:3, 1:4, 1:6, 1:8, 1:12 and 1:16 dilutions of the fluid to be tested. Titrate the complement and add 2 full units (1 cc.); also 0.5 cc. of saline solution.
3. Water bath at 37° C. for 1 hour.
4. Add 2 units of hemolysin and 0.5 cc. of 2 per cent corpuscles.
5. Water bath 1 hour and read.
6. Include a hemolytic system and corpuscle control.

The smallest amount giving even slight interference of hemolysis is the anticomplementary unit and varies greatly with different specimens.

7. In setting up the complement fixation tests, place  $\frac{1}{4}$  of the anticomplementary unit in a front and rear tube; for example, if this happens to be 0.5 cc. of 1:4 dilution, use 0.5 cc. of 1:16.

8. To the front tube add the usual dose of antigen and to the rear tube 0.5 cc. of saline (control).

9. The complement is titrated in the presence of the antigen in the usual manner and 2 full units added to both tubes.

10. The primary incubation is the usual 15 to 18 hours at 6° to 8° C. followed by 10 to 15 minutes in a water bath, the test being finished in the usual way.

### KOLMER COMPLEMENT FIXATION TESTS FOR PROTOZOAL AND METAZOAL DISEASES

Methods for preparing the various antigens are given below. The methods for titrating the hemolytic, anticomplementary and antigenic activities are exactly as given for the titration of bacterial antigens, the hemolytic system and general technic being exactly as described for syphilis.

Each antigen is employed in a dose equivalent to  $\frac{1}{3}$  or  $\frac{1}{4}$  of its anticomplementary unit since the large amounts yield the most sensitive reactions, prezone reactions being quite uncommon. Therefore the anticomplementary unit of each antigen must be known on the basis of preliminary titrations.

In conducting the main tests, either the quantitative or qualitative technic may be employed with a primary incubation of 15 to 18 hours in a refrigerator at 6° to 8° C. followed by  $\frac{1}{2}$  hour in a water bath at 37° C., or the water bath only may be employed for 2 hours; the former has yielded the more sensitive and satisfactory results.

*In conducting the echinococcus and other tests with human sera, it is advisable (indeed necessary) to conduct a Wassermann test at the same time because all of these antigens are capable of yielding cross complement fixation reactions with sera containing large amounts of syphilis antibody. Whenever a serum gives a positive Wassermann reaction, the results of positive echinococcus or other reactions should be interpreted with due care.*

When rabbit immune sera are used for positive controls, the dose should not be more than 0.5 cc. of 1:50 dilution (0.01 cc. serum) in order to avoid the nonspecific reactions which are sometimes yielded by normal rabbit serum with these antigens.

**Endamoeba Histolytica Antigen.**—Antigen may be prepared according to the method of Craig by extracting cultures of the parasite or mucoid material obtained from the intestine of infected dogs, rich in the amebae, with 7 or 8 volumes of absolute alcohol and extracting for 15 days at 37° C. with daily shaking; after filtration the filtrate is used.

To eliminate numerous contaminating bacteria and their metabolic products occurring in culture antigens, Rees and his associates (*Am. Jour. Trop. Med.*, Nov., 1942) have described a method in which *E. histolytica* is cultivated in a fluid medium with a single bacterial symbiont designated as "organism t" and probably *Leptotrichia buccalis*. After 72 hours of incubation the amebae are recovered from the fluid by centrifugation, washed with Locke's solution and suspended in Locke's solution. The

antigen is then frozen for 4 hours in dry ice and thawed overnight at 10° C. It is then cleared of sediment by centrifugation in the angle head machine and stored at 10° C.

**Malarial Antigen.**—According to the *method of Coggeshall and Eaton (Jour. Exper. Med.* 67: 871, 1938; *ibid.*, 69: 379, 1939) antigen may be prepared of the blood of monkeys dying of infection with *P. knowlesi*. Blood containing 20 to 50 per cent of parasitized erythrocytes is collected in 2 per cent sodium citrate solution, centrifuged to separate the serum, and washed twice with saline solution. The packed cells and parasites are then suspended in an equal volume of saline, frozen, dried and ground. The equivalent of each 1 cc. of packed cells is suspended in 10 cc. of saline solution, frozen and thawed four times, centrifuged, and the supernatant used as antigen.

**Leishmania Antigen.**—The 3 species of leishmania may be cultivated on the blood medium of Senekji prepared as follows: Bacto beef extract, 50 parts is dissolved in distilled water 1000 parts and heated at 50° C. for 1 hour followed by 80° C. for 5 minutes. The solution is then passed through filter paper and the following ingredients added: 20 parts neopeptone, 20 parts agar (Nobel) and 5 parts chemically pure sodium chloride. Adjust to a pH of 7.2 to 7.4 and autoclave at 121° C. for 20 minutes. Cool to 45° C., add sterile defibrinated rabbits' blood to make 10 per cent of the medium and slant. Antigens are prepared by suspending cultures of leishmania in saline solution containing 1 per cent glycerine and 0.5 per cent phenol.

**Trypanosome Antigens.**—Antigen may be prepared of *T. cruzi* for complement fixation tests in Chagas' disease by cultivating the parasite on the blood medium of Senekji and preparing suspension in saline solution containing 1 per cent glycerine and 0.5 per cent phenol.

In complement fixation tests with the sera of horses for dourine, white rats are inoculated and as soon as the tail blood shows the presence of a heavy infection, antigens are prepared by securing the organisms from the blood by the method of Reynolds and Schoening as follows: "Blood of infected rats is collected in a 1 per cent sodium citrate solution in physiological salt solution in order to prevent coagulation. When all the blood has been collected, the solution is filtered through cheesecloth to remove clots, fibrin, etc., poured into tubes, and centrifugalized for about 20 minutes at 2100 revolutions per minute. This precipitates all the corpuscles and most of the trypanosomes, leaving an upper stratum of blood serum and citrate solution containing some of the organisms. This fluid is drawn off and again centrifugalized in order to recover any of the protozoa which may be present. To the other tubes containing the mass of corpuscles intermixed with and superimposed by trypanosomes is added sufficient distilled water to produce complete hemolysis of the rat erythrocytes, a matter of about 20 minutes, which procedure is facilitated by agitation of the mixture in a flask. This also is centrifugalized but in this instance for about  $\frac{1}{2}$  hour, upon the completion of which there is found at the bottom of the tubes a mass of trypanosome with an admixture of stroma of the hemolyzed red cells, which latter, in quantity, has been found to be negligible. After discarding the supernatant fluid (hemoglobin-stained water) physiological salt solution is added and the material vigorously shaken until the mass of trypanosomes is disintegrated and evenly distributed throughout the solution. Centrifuging is again resorted to with similar results, the washed mass of trypanosomes being packed at the bottom of the tubes. The salt solution is poured off and an amount of preserving fluid (physiological salt solution and glycerin) equal to



about twice the amount of trypanosomes added; the mixture is then agitated until a uniform suspension is acquired, when it is stored in a refrigerator at a low temperature until used."

**Echinococcus Antigen.**—The fluid from echinococcus cysts has been usually employed preserved with 0.5 per cent phenol in a refrigerator. It would appear, however, that the scolices contain most of the antigenic principles and a better antigen may be prepared by grinding up the moist scolices with fine sand in a mortar and adding 9 volumes of the clear cyst fluid or saline solution to give an approximate 10 per cent extract of the scolices. Phenol or tricresol should be added to 0.25 per cent and the mixture extracted in an incubator at 37° C. for 4 days, filtered, and stored in a refrigerator.

Fairley recommends an alcoholic extract prepared by grinding the scolices with fine sand, adding 9 volumes of absolute ethyl alcohol to give a 10 per cent extract, and placing the mixture in an incubator for 2 days when it is filtered and stored for use.

**Schistosomiasis Antigen.**—This antigen, which is group specific, is usually prepared according to the *method of Fairley* by extracting each infected snail liver with 1 cc. of absolute alcohol at 37° C. for 24 hours with shaking. The extract is then filtered, the filtrate dried and the antigen completed by dissolving 0.1 gm. in 4 cc. normal saline solution. An alternate method consists in concentrating the filtrate in a water bath at 45° C. to the point of turbidity, adding just enough absolute alcohol to clarify it, and dispensing in 1 cc. ampules which are kept in the refrigerator. When needed, 1 cc. is diluted with 39 cc. normal saline solution.

**Paragonimiasis Antigen.**—This antigen may be prepared by extracting macerated adult *Paragonimus westermani*, obtained from human cases at autopsy or from experimentally infected laboratory animals, with 0.5 per cent solution of phenol in normal saline solution at 37° C. for several days.

### KOLMER COMPLEMENT FIXATION TEST FOR BLOOD STAINS

**Preparation and Titration of Extracts.**—1. If the material for testing is a stain on a fabric like a towel, handkerchief, undergarment, etc., a portion is carefully cut away with clean scissors and extracted in sterile phenolized saline solution prepared by diluting 1 cc. of pure phenol up to 400 cc. with saline (0.25 per cent and sufficient for preventing bacterial growths). If it appears to be an unwashed stain of blood a piece or aggregate of pieces totalling about 2 inches square may be extracted in 50 cc. of phenolized saline solution. If the stain has been partially washed out, correspondingly larger pieces should be extracted or smaller amounts of phenolized saline employed. Sometimes it is advisable to tear portions into single threads which may show incrustations, especially if the fabric is of a heavy kind. Spots may be likewise scraped from wood, metal and plastered walls with *clean* scalpels and collected in containers or placed at once in phenolized saline solution. As a general rule each 0.1 gram of material should be extracted in about 10 cc. of sterile solution to obtain approximately 1 to 100 extracts. One part of cloth should be extracted in 100 parts of sterile phenolized water to give complete hemolysis and later rendered isotonic by adding 1 gram of sodium chloride to each 100 cc. *In medicolegal work it is imperative to exercise every precaution against confusion and the source of every specimen, along with other essential data, should be carefully recorded.*

2. It is well to prepare *control extracts* at the same time and in the same manner by extracting similar pieces of *unstained* fabric or other material if available, which is usually the case.

The extracts should be placed in an incubator for 24 hours, or longer, for the solution of flinty particles from spots on metal or wood. Heating the extracts at about 40° C. for an hour or two sometimes hastens solution. Sometimes it is necessary to promote solution by extracting with 0.5 per cent solutions of sodium hydroxide in saline solution and later neutralizing with decinormal hydrochloric acid with phenolphthalein indicator.

3. Small portions of blood-soaked plaster, earth, hay, straw, grass, leaves, etc., may be extracted with phenolized saline solution but in the case of leather, wood, bark and earth the reaction should be taken and the extracts brought to the neutral point.

4. The extracts should be filtered through paper if necessary although in most instances they can be used unfiltered since they do not require the crystal clearness essential for the precipitation test.

5. Each extract should now be tested chemically for blood by the phenolphthalein, benzidine or some other reliable method. If these or tests applied directly to the material are negative for blood it is hardly advisable to go on with the complement fixation tests.

6. Determine the anticomplementary unit of each extract as shown in Table 48.

**Preparation and Titration of Immune Serum.**—1. *It is essential to use immune serum containing large amounts of antibody* in order to secure specific and reliable results; this is especially true in medicolegal tests involving the charge of murder and the unit should not be less than 0.5 cc. of 1 to 200 of serum when titrated as described below.

2. Large, healthy and previously unused rabbits are usually chosen and any one of several methods of immunization may be employed. One of the best methods is the daily intravenous injection of 0.1 cc. of sterile defibrinated blood or serum diluted with 0.9 cc. of sterile saline solution for 21 to 28 injections. By exercising due care with the ear veins no difficulties are encountered, the mortality is low and the yield of antibody usually quite good.

3. A second method is to give 0.5 cc. of sterile defibrinated blood or serum diluted with 4.5 cc. of sterile saline solution by intravenous injection every 5 to 7 days for 6 to 8 injections.

4. One week after the last injection by either method some blood is removed from the ear of each animal and a titration made of the antibody content as described below. If the unit is 0.5 cc. of 1 to 200 or higher (preferably 1 to 500 to 1 to 1000) the animal is bled, the serum separated and preserved with an equal part of chemically pure glycerin.

5. Dilute 0.1 cc. of serum with 0.9 cc. of saline solution and heat at 62° C. for 30 minutes; add 19 cc. of saline solution (1:200). If the serum is preserved with an equal part of glycerin dilute 0.2 cc. with 0.8 cc. saline solution, heat and add 19 cc. saline solution (1:200).

6. Prepare an antigen of homologous blood by diluting 1 cc. of defibrinated blood in 100 cc. of sterile water and after complete hemolysis add 1 gram of sodium chloride. Or dissolve 0.2 gram of pulverized dried blood in 20 cc. of sterile saline solution. Titrate for anticomplementary activity and use in dose of  $\frac{1}{2}$  the unit

**TABLE 48.—THE ANTICOMPLEMENTARY TITRATION OF A BLOOD STAIN EXTRACT**

Tube	Extract 0.5 cc.	Complement 2 full units	Saline	Refrigerator at 6 to 10° C. for 15 to 18 hours, followed by water bath for 10 to 15 minutes.		Hemolysin 2 units	Corpuscles 2 per cent	Water bath one hour.		Readings
1	Undiluted	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Incomplete hemolysis (unit) *
2	1 to 2	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
3	1 to 3	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
4	1 to 4	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
5	1 to 6	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
6	1 to 8	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
7	1 to 12	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
8	1 to 16	1.0 cc.	0.5 cc.			0.5 cc.	0.5 cc.			Complete hemolysis
9	—	1.0 cc.	1.0 cc.			0.5 cc.	0.5 cc.			Complete hemolysis (control).
10	—	—	—			—	0.5 cc.			No hemolysis (control).

\* Anticomplementary unit 0.5 cc. of undiluted extract. The doses for the complement fixation tests would be 0.5 cc. of 1 to 2, 1 to 4, 1 to 6, 1 to 8, 1 to 12 and 1 to 16. If this tube showed complete hemolysis (absence of anticomplementary activity) the doses would be 0.5 cc. undiluted, 1 to 2, 1 to 3, 1 to 4, 1 to 6 and 1 to 8 dilutions.

although these antigens are almost invariably without anticomplementary activity and may be used in a dose of 0.5 cc. of 1 to 2 dilution of the stock 1 to 100 solution.

7. Complete the test as shown in Table 49. *The smallest amount of immune serum giving a ++++ reaction is the unit* and the bottle or vial may be labelled accordingly. If the serum is titrated 1 or 2 weeks after ripening in a refrigerator, the unit remains quite constant over months of time and the serum may be used without retitration. *But in medicolegal cases it is always advisable to retitrate* before setting up the complement fixation tests. *The amount employed in the complement fixation tests is 2 to 10 units in 0.5 cc. providing this amount does not exceed 0.5 of a 1 to 100 dilution.* If the unit were just 0.5 cc. of 1 to 200 the dose would be 2 units or 0.5 cc. of 1 to 100. If the unit is less than 0.5 cc. of 1 to 200 the serum should not be used.

**Test.**—1. The unknown extract is used in 6 different doses. If the stock solution is not anticomplementary when titrated as shown in Table 48 the doses are 0.5 cc. of stock and 0.5 cc. of 1 to 2, 1 to 3, 1 to 4, 1 to 6 and 1 to 8 dilutions of it (Tubes 1, 2, 3, 4, 5 and 6).

2. If control extracts are included they may be prepared by dissolving 0.5 cc. of defibrinated blood in 50 cc. of sterile water and adding 0.5 gram of sodium chloride. As a general rule they do not require titration and may be used in a single dose of 0.5 cc. of 1 to 2 dilutions as shown in Tubes 10, 11, 12, 16, 17 and 18 of Table 50.

3. The hemolysin is titrated as described on page 675.

4. The complement is titrated in the presence of the *largest amount of each blood extract employed* and used in a dose of 2 full units. The general arrangement is the same as shown on page 677 except that the dose of antigen is usually 0.5 cc. of undiluted or 1 to 2 dilution of stock solution of each extract.

5. The antihuman and other antisera are heated at 62° C. for 30 minutes and used in a dose of 2 to 10 units providing this dose is not more than 0.5 cc. of 1 to 100 of serum. If the unit happens to be 0.5 cc. of 1 to 200, 2 units or 0.5 cc. of 1 to 100 may be used. But if the unit is less than 0.5 cc. of 1 to 200 the serum should not be used.

6. When extracts and antisera are mixed there may be an interval of 10 minutes before the addition of complement although this is not essential.

7. Each extract is controlled for anticomplementary activity and each should show complete hemolysis, as shown in Tubes 7, 16, 17 and 18.

8. Each antiserum should be controlled for anticomplementary activity and give complete hemolysis, as shown in Tubes 13, 14 and 15.

9. A positive control employing an extract of human blood and antihuman serum should be included and show a strongly positive reaction, as in Tube 10.

10. Likewise controls on each known extract set up with homologous sera as shown in Tubes 11 and 12 and giving strongly positive reactions.

11. A hemolytic system control should be included and give complete hemolysis (Tube 19).

12. A corpuscle control should show no hemolysis (Tube 20).

**TABLE 49.—TITRATION OF ANTISERUM**

Tube	Antiserum 0.5 cc.	Blood antigen *	Complement 2 full units	Refrigerator at 6° to 10° C. for 15 to 18 hours followed by water bath for 10 to 15 minutes.		Hemolysin 2 units	Corpuscles 2 per cent	Water bath one hour.		Readings
1	1 to 200	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++ (prezone)
2	1 to 300	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++++
3	1 to 400	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++++
4	1 to 600	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++++
5	1 to 800	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++++ (unit)
6	1 to 1200	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++++ (unit)
7	1 to 1600	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			++
8	1 to 2400	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			+
9	1 to 3200	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			+
10	1 to 4800	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			+
11	0.5 cc. saline	0.5 cc.	1.0 cc.			0.5 cc.	0.5 cc.			— (antigen control)
12	1.0 cc. saline	—	1.0 cc.			0.5 cc.	0.5 cc.			— (hemolytic control)

\* Dose  $\frac{1}{2}$  of anticomplementary unit; usually 0.5 cc. of 1 to 3 dilution of stock 1 to 100 solution.

Highest dilution or smallest amount of serum giving ++++ reaction and therefore the unit. In the main tests 10 units or 0.5 cc. of 1 to 120 dilution would be used. If all of the first 10 tubes show ++++ reactions with negative controls, higher dilutions of immune sera would require testing since the unit would be less than 0.5 cc. of 1 to 4800.

**TABLE 50.—THE COMPLEMENT FIXATION TEST FOR THE IDENTIFICATION OF A BLOOD STAIN**

Tube	Extracts 0.5 cc. of:	Heated anti- serum 0.5 cc. carrying 2 to 10 units	Complement (2 full units)		Hemolysin (2 units)	Corpuscles (2 per cent.)	Water bath one hour. Results.
1	Unknown stock solution *	Antihuman	1.0 cc.	Refrigerator incubation at 6° to 10° C. for 15 to 18 hours followed by 10 to 15 minutes in a water bath at 37° C.	0.5 cc.	0.5 cc.	+++ **
2	Unknown 1 to 2 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
3	Unknown 1 to 3 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
4	Unknown 1 to 4 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
5	Unknown 1 to 6 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
6	Unknown 1 to 8 of stock	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	+++
7	Unknown stock solution *	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	++
8	Unknown 1 to 2 of stock	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	— (antigen control)
9	Unknown 1 to 2 of stock	Antiox	1.0 cc.		0.5 cc.	0.5 cc.	— (specificity control)
10	Human blood 1 to 2 of stock φ	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	++++ (positive control)
11	Dog blood 1 to 2 of stock φ	Antidog	1.0 cc.	Refrigerator incubation at 6° to 10° C. for 15 to 18 hours followed by 10 to 15 minutes in a water bath at 37° C.	0.5 cc.	0.5 cc.	++++ (control)
12	Beef blood 1 to 2 of stock φ	Antiox	1.0 cc.		0.5 cc.	0.5 cc.	++++ (control)
13	0.5 cc. saline	Antihuman	1.0 cc.		0.5 cc.	0.5 cc.	— (serum control)
14	0.5 cc. saline	Antidog	1.0 cc.		0.5 cc.	0.5 cc.	— (serum control)
15	0.5 cc. saline	Antiox	1.0 cc.		0.5 cc.	0.5 cc.	— (serum control)
16	Human blood 1 to 2 of stock *	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	— (antigen control)
17	Dog blood 1 to 2 of stock *	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	— (antigen control)
18	Beef blood 1 to 2 of stock *	0.5 cc. saline	1.0 cc.		0.5 cc.	0.5 cc.	— (antigen control)
19	1.0 cc. saline	—	1.0 cc.		0.5 cc.	0.5 cc.	— (system control)
20	2.5 cc. saline	—	—		—	0.5 cc.	No hemolysin (corpuscle control)

\* Not anticomplementary in 0.5 cc. of stock solutions but each extract is controlled in the maximum amounts employed in tubes 7, 16, 17 and 18.

φ Prepared by dissolving 0.5 cc. of defibrinated blood in 50 cc. of water and adding 0.5 gm. of sodium chloride.

\*\* Strongly positive; — = complete hemolysis (negative), etc.

## METHODS FOR CONDUCTING FLOCCULATION AND PRECIPITATION TESTS

Numerous precipitation or flocculation tests have been described for the serum diagnosis of syphilis by Michaelis, Sachs-Georgi, Vernes, Meinicke, Dreyer and Ward, Kahn, Kline, Eagle, Hinton, Rosenthal, Mazzini, Boerner, Jones and Lukens, and others. Their value in comparison to the Wassermann test depends not so much on their infallibility as on the fact that the macroscopic and microscopic flocculation tests afford a different approach to the serum diagnosis of syphilis and to the serological guidance of treatment.

### BOERNER, JONES AND LUKENS' MICROSCOPIC AND MACROSCOPIC FLOCCULATION TESTS FOR SYPHILIS \*

**Preparation of Antigen.**—1. To 10 grams of powdered beef heart (Difco) add 20 volumes of acetone (200 cc.) and shake frequently for at least 30 minutes by hand, or shake continuously on shaking machine for at least 5 minutes.

2. Filter through paper. Wash with 50 cc. of fresh acetone, and discard the filtrate.

3. Dry the powder to absence of acetone odor.

4. Place the powder in a flask and add 30 volumes of alcohol-ether mixture (225 cc. of absolute alcohol and 75 cc. of ether U.S.P.). Allow to stand at room temperature for  $\frac{1}{2}$  hour, mixing thoroughly every 5 minutes or shake continuously on shaking machine for 15 minutes.

5. Filter through filter paper and discard the residue.

6. Place the filtrate in a large flask or beaker and evaporate to 10 cc. by boiling in a water bath or on a hot plate. If the concentration is below 10 cc., add sufficient absolute alcohol to make up to this amount.

7. Place the concentrated filtrate in a well-stoppered flask or bottle, and place in the refrigerator at 6° C. to 8° C. overnight.

8. Filter through a good grade filter paper. This filtration should be done in the refrigerator so that the filtration will be completed while the solution of antigen is still cold.

9. Allow to stand overnight at room temperature, and if any precipitate forms, it should be filtered or centrifuged out. Store in amber glass bottle at room temperature. If a slight precipitate forms after storage, it should be removed by filtering or centrifuging. This precipitation does not alter the usefulness of the antigen. Larger amounts may be prepared by proportional increase in the amount of beef heart and solvents.

**Preparation of Antigen Emulsion.**—1. Arrange 4 test tubes in a row (Kolmer tubes are very satisfactory).

2. In the first tube, place 2 cc. of saline solution (0.85 per cent).

3. In the second tube, place 0.1 cc. of antigen (pipet to the bottom of the tube for greater accuracy).

4. In the third tube, place 1.0 cc. of distilled water (the water should be boiled for 5 minutes and then cooled just before use).

5. In the fourth tube, place 1.0 cc. of 1 per cent cholesterol in absolute alcohol,

\* Boerner, F., Jones, C. A., and Lukens, M.: Simplified Microscopic and Macroscopic Flocculation Tests for the Diagnosis of Syphilis. *Am. Jour. Clin. Path.*, 10: 141, 1940.

heated to 37° C. before use to insure complete solution of the cholesterol. A supply of cholesterolized alcohol may be prepared and kept at 37° C.

6. Pour quickly the contents of tube 4 into tube 3; immediately pour the contents of tube 3 into tube 2; immediately pour the contents of tube 2 into tube 1. Place a stopper in tube 1 and shake vigorously for 1 minute.

7. Place the antigen in a water bath or incubator at 37° C. for 15 minutes.

8. This emulsion should be prepared fresh for each day's tests. If more than 4 cc. is required, several lots may be prepared and then pooled.

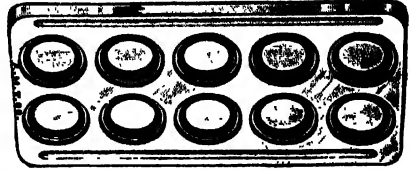


FIG. 301.—BOERNER MICRO TEST SLIDE

**Microscopic Test.**—1. Prepare emulsion of antigen as previously described.

2. Inactivate the serum to be tested at 56° C. for ½ hour, or at 58° C. for 10 minutes.

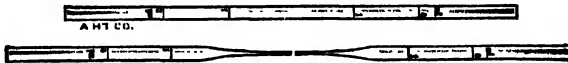


FIG. 302.—BOERNER ANTIGEN EMULSION PIPET

3. Special slides are recommended for use in this test (Fig. 301). These should be clean and fat free. Immediately after use the slides should be placed in water. At a convenient time they are removed and scrubbed with soap and water, and rinsed

with tap water. They are then placed in absolute or 95 per cent alcohol where they can remain until ready for use (any glass or metal container that can be covered tightly may be used). Just before using, remove from the alcohol and wipe dry with gauze or cloth. A plain slide may be used in which case rings about 12 to 13 mm. inside diameter are made with wax pencil or paraffin.

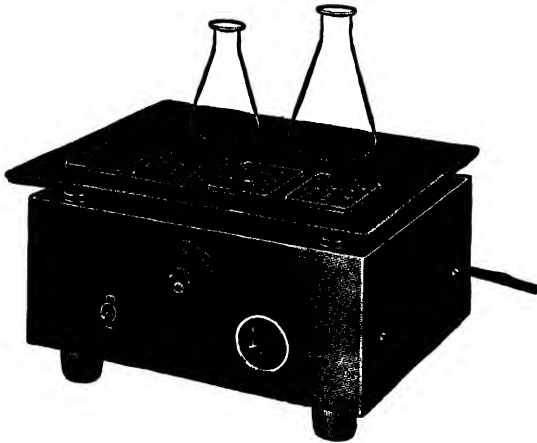


FIG. 303.—ELECTRIC ROTATING APPARATUS

4. Place 0.1 cc. or 0.05 cc. of serum to be tested in the ring. (The sensitivity is slightly decreased when the smaller dose of serum is employed.)

5. Add 1 drop of the antigen (0.01 cc.) from a capillary pipet. Pipet should be calibrated to deliver between 95 and 105 drops per cc. A special pipet is recommended which is easily made and calibrated (Fig. 302). A syringe with a needle attached which will deliver the required number of drops may also be used.

6. Place slide on rotor (Fig. 303), and allow to shake for 5 minutes or rotate slide on flat surface for 5 minutes. If the oscillating platform shaking machine is used, the time should be 10 minutes.



7. Examine immediately under low power magnification (16 mm. objective and eyepiece 10).

8. Negative tests will show uniform dispersion or small granules. Positive reactions show varying degrees of aggregation and may be reported as plus 4, plus 3, plus 2 and plus 1. Doubtful reactions show indefinite aggregation of the particles, and in such cases it is advisable to repeat the test. If the repeated test gives a plus 1 or doubtful reaction, it should be reported as doubtful. If it is negative, then it should be reported as negative.

**Macroscopic Test (Serum).**—1. Prepare emulsion of antigen as previously described.

2. Inactivate the serum to be tested at 56° C. for ½ hour, or for 10 minutes at 58° C.

3. One tube is used for each test (Kahn tube or one of similar diameter). (A special wire rack has been designed for use in this test, especially useful where a large number of tests are run (Fig. 304).

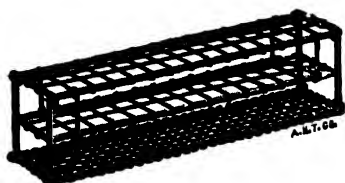


FIG. 304.—BOERNER TEST TUBE SUPPORT

4. Place 0.25 cc. of serum in the tube.

5. Add 0.05 cc. of antigen emulsion to each tube.

6. Shake the tube on shaking machine (Fig. 305), or vigorously by hand for 5 minutes.

7. Centrifuge at high speed for 10 minutes (2000 r.p.m.).

8. Add 1 cc. of saline (0.85 per cent).

9. Resuspend by tapping bottom of tube gently and read.

10. Those showing definite flocculation are reported as positive. Those showing no flocculation are reported as negative. Those showing indefinite flocculation or questionable flocculation should be repeated and if a very weakly positive or doubtful reaction is obtained, it should be reported as doubtful. If negative on second test, it should be reported as negative. In all such cases, the serum should be carefully examined for foreign particles before retesting.

**Macroscopic Test (Spinal Fluid).**—1. Prepare emulsion of antigen in same manner as previously described, and dilute with an equal volume of 0.85 per cent saline.

2. Place 1.0 cc. of spinal fluid in a Kahn tube or one of similar size.

3. Heat at 56° C. for ½ hour, unless previously heated for other tests.

4. Add 0.05 cc. of antigen emulsion, and mix by gentle shaking.

5. Incubate at 6° C. to 8° C. for 15 to 18 hours.

6. Centrifuge 10 minutes at high speed (2000 r.p.m.).

7. Resuspend by tapping bottom of tube gently and read the degree of flocculation as described above in the test with blood serum.

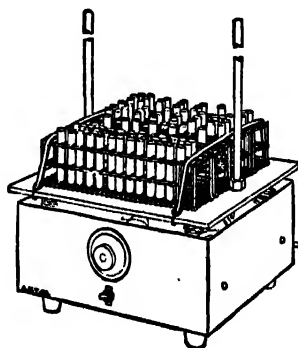


FIG. 305.—BOERNER SHAKING APPARATUS

**EAGLE, MACROSCOPIC AND MICROSCOPIC TESTS FOR SYPHILIS**

**Preparation of Serum.**—The clot is centrifuged, and 0.1 cc. of the clear supernatant serum is transferred to a numbered tube. Thick-walled test tubes without lip, measuring 9 to 12 mm. in outside diameter, and 75 to 90 mm. in length, will prove satisfactory. With the larger tubes, it will be found convenient to work with larger quantities, and 0.2 cc. or 0.4 cc. of the serum may be used, with a correspondingly larger amount of antigen.

It is essential that the serum be free of red blood cells or suspended particles which might simulate lipoidal aggregates. The use of strongly hemolyzed serum does not affect the results save insofar as it makes it difficult to recognize aggregates with the naked eye. However, the results can usually be read with no difficulty under the microscope. The serum is inactivated by heating at 56° C. for 20 minutes, or at 60° to 62° C. for 3 minutes.

**Preparation of Antigen.**—Fifty grams of dried powdered beef heart (Difco) are extracted for 15 minutes at 30° to 37° C. with 250 cc. of anesthesia ether, with frequent shaking. The mixture is filtered with suction, the ether extract is discarded, and the powder is similarly extracted with a second portion of fresh ether (250 cc.). This is repeated for a total of 4 extractions. All the ether extracts are discarded. The beef heart powder is then washed on the filter with 100 cc. of fresh ether, thoroughly dried, and extracted with 250 cc. of absolute ethyl alcohol for 3 to 5 days at 20° to 37° C. The flask should be periodically shaken during that interval. At the end of this time the alcohol mixture is filtered, and the moist powder is washed with small portions of fresh absolute alcohol until the combined alcoholic extract and washings measure 250 cc.

This basic extract is fortified with 0.6 per cent each of cholesterol and corn germ sterol (6 mg. of each sterol per cc. antigen). The required amount of sterol is added to a measured volume of antigen and is dissolved by boiling. One cubic centimeter of this completed antigen suffices for 500 tests.

Each lot of antigen should be checked by performing several tests with known negative and weakly positive serums. The antigen remains serviceable for years if stored at room temperature in tightly stoppered containers.

**Preparation of Antigen Dilution.**—One volume of the fortified antigen is diluted with 2 volumes of 0.85 per cent ("physiologic") NaCl solution. The measured amount of salt solution is blown into the antigen with a pipette. The tip of the pipette should have a sufficiently large bore to make the admixture of salt and antigen rapid and complete. The milky suspension which forms is the stock antigen dilution, which should be aged for at least 24 hours in the ice box before being used. Stored in the ice box, this primary dilution remains serviceable for at least 5 days.

For actual use in the test, a measured amount of this stock dilution (*e.g.*, 0.2 cc.) is further diluted with 8 volumes (*e.g.*, 1.6 cc.) of 4 per cent NaCl. This second dilution should be made just before the antigen is added to the serum.

**Routine Serum Test.**—One volume of the freshly diluted antigen (*i.e.*, the stock dilution freshly diluted with 8 volumes of 4 per cent NaCl) is added to 2 volumes of inactivated serum. Thus, to 0.1 cc. serum add 0.05 cc. of the antigen suspension; to 0.4 cc. serum add 0.2 cc. of the antigen suspension. The serum-antigen mixture is

vigorously shaken for 5 minutes. If a shaking machine is used, it should be adjusted to approximately 180 to-and-fro movements<sup>1</sup> per minute, with a 4-cm. stroke. The tubes are then placed in a 37° C. water bath for 30 minutes (may be omitted if the tubes have been shaken in a mechanical shaker for 5 minutes). At the end of this time, they are centrifuged at 1500 to 2000 r.p.m. for 10 minutes. Prolonged centrifugation at excessive speeds is to be avoided, as it causes partial aggregation of the antigen crystals and false doubtful reactions on microscopic examination.

The results are read at once, by either macroscopic or microscopic observation, or both.

**Emergency Serum Test.**—In case of emergency, as for a blood transfusion, the entire test can be completed within 20 to 25 minutes. The serum is inactivated for 3 minutes at 60° to 62° C., antigen is added as in the routine test, and the tube is shaken for 5 minutes in a shaking machine at 180 complete to-and-fro movements per minute with a 4-cm. stroke. The incubation is omitted. The tube is then centrifuged for 8 minutes at 1500 to 2000 r.p.m., and the results are read as in the routine test, to be now described.

**Reading of Results.**—In general, aggregates are easier to see under the microscope than with the naked eye. It follows that very weakly positive serums may appear doubtful or even negative when viewed macroscopically, and yet the serum-antigen mixture may show unmistakable aggregation by microscopic examination. Whenever feasible, it is therefore recommended that the tests be read both macroscopically in the tube, and microscopically by pouring a little of the serum-antigen mixture onto a glass slide. If the number of tests makes this prohibitively laborious, it is suggested that the microscopic examination be used wherever there is the slightest doubt as to the macroscopic reading. Almost all otherwise doubtful reactions are thus resolved into positive or negative.

(a) *Macroscopic Reading.*—In a positive result, a few coarse aggregates are seen floating in a comparatively clear fluid. In a few positive serums, the fluid is water-clear and the aggregates have sedimented to form a coherent flake at the bottom of the tube. If the aggregation is questionable, the result is doubtful. In a negative result, the tube at rest seems opalescent and homogeneous; when it is gently shaken, a swirl of the refractile crystals of the antigen is seen. Occasionally, these discrete crystals are floated to the top of the serum on centrifugation; these immediately redisperse on mild agitation and are readily differentiated from the coherent aggregates of a positive result. Vigorous shaking of the contents of the tube is to be avoided, as sedimented dirt particles of red blood cells are thus resuspended.

(b) *Microscopic Reading.*—The contents of the tube are poured onto a glass slide. It is convenient to use hollow ground slides, with depressions 1.5 cm. in diameter and 1.5 to 2 mm. deep. If these are not available, circles 2 cm. in diameter are inscribed on ordinary slides with a glass marking pencil. It is essential that not more than 2 minutes elapse between the time the serum-antigen mixture is poured on the slide and its examination under the microscope. The magnification should be about 100-fold (10X ocular, 16 mm. objective). The light should be adjusted so that a control negative serum appears homogeneous and should be kept in that position while the actual tests are read.

In a positive result, irregular aggregates of varying degrees of coarseness are seen floating in a clear fluid. If the aggregation is questionable, the result is doubtful. In

a negative result, a uniform distribution of minute and discrete antigen crystals is seen over the entire field.

**Reporting the Results.**—In reporting the results to the physician, if the macroscopic reading is positive or doubtful and the microscopic reading is positive, the test is reported as "Positive."

If the macroscopic reading is negative, but unmistakable aggregates are seen under the microscope, the test is none the less positive. If there is sufficient serum for a repeat test, such discrepancies should be checked before being reported. If the result has been so confirmed by a repeat test, the report should include a statement to that effect ("Positive—confirmed by duplicate test").

If the microscopic examination shows indefinite aggregation the macroscopic reading is almost invariably negative, and the test is reported as "Doubtful. Please repeat." If there is sufficient serum for retesting, such results should be checked before being reported. The report should include a statement to that effect ("Doubtful—confirmed by duplicate test. Please repeat").

**Quantitative Serum Flocculation Test.**—If it is necessary or desirable to ascertain the degree of positivity of a known positive serum, this can be done by carrying out the routine test on serial dilutions of serum as shown in the following table:

Whole inactivated serum, cc. . . .	0.2	0.1	0.05						
Serum, diluted 1:8 with 0.85 per cent NaCl, cc. . . . .		0.2	0.1	0.05					
Serum, diluted 1:64 with 0.85 per cent NaCl, cc. . . . .						0.2	0.1	0.05 etc.	
0.85 per cent, NaCl, cc. . . . .	0	.1	.15	0	.1	1.5	0	.1	.15
Final dilution of serum . . . . .	1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Result of test . . . . .	+	+	+	+	+	+	±	0	0

The serum is positive up to a 1:48 dilution, a reagin titer of 48. Very rarely, zone reactions are observed in which strongly positive serums give a negative result in the routine test with whole serum but are strongly positive if tested in a 1:10 or 1:20 dilution.

**Spinal Fluid Test.**—Although the ice box Wassermann technic is to be preferred for the examination of spinal fluids because of its greater sensitivity, the microfloculation procedure gives satisfactory, even if not optimum, results.

To 1.0 cc. of fresh fluid are added 0.1 cc. of the final antigen dilution, prepared as already described for the serum flocculation test. The mixture is shaken, centrifuged, and read as previously described.

The test can be performed quantitatively by using graded quantities of spinal fluid as indicated in the following table:

Spinal fluid, cc. . . . .	1.0	0.4	0.2	0.1	0.05
0.85 per cent NaCl, cc. . . . .	0	.6	.8	.9	.95
Antigen suspension, cc. . . . .	.1	.1	.1	.1	.1

### HINTON MACROSCOPIC FLOCCULATION TEST FOR SYPHILIS (THIRD MODIFICATION)

This test requires precision in execution because consistent and accurate results cannot be obtained if minor variations in technic are allowed to creep into the routine.

**Apparatus.**—1. Test-tube racks. To simplify numbering and pipetting sera, these racks should be constructed to hold 10 or 20 tubes in a row.

2. Serum tubes 100 mm. long with an approximately uniform inside diameter of 10 mm.

3. A water bath for inactivating sera.

4. A Wassermann bath or a bacteriologic incubator. The former is preferred, because by its use the test is from 3 to 5 per cent more sensitive. The water in the bath should be changed frequently to prevent a deposit from sticking to the outside of the tubes.

5. A centrifuge with a speed of over 2000 revolutions per minute.

6. A thermometer that registers the maximum and minimum temperatures.

7. Graduated 100 cc. and 250 cc. cylinders for measuring the reagents.

8. Dropping pipets with rubber bulbs of about 5 cc. capacity, for drawing off sera.

9. Serologic pipets of 1.0 cc. capacity, graduated in tenths to the tip, for measuring the serums, and 5 cc. or 10 cc. serologic pipets for measuring the reagents.

10. Thick-walled Erlenmeyer flasks of 125 or 250 cc. capacity, with an inverted V-shaped ridge blown in the bottom (Fig. 306), for mixing glycerinated indicator. This ridge should produce 2 semicircular compartments, each of which should hold from 3 to 5 cc. in flasks with a capacity of 125 or 250 cc. (Flasks of this type are not listed in any of the catalogues. They may be obtained from Macalaster Bicknell Company of Boston, Massachusetts.)

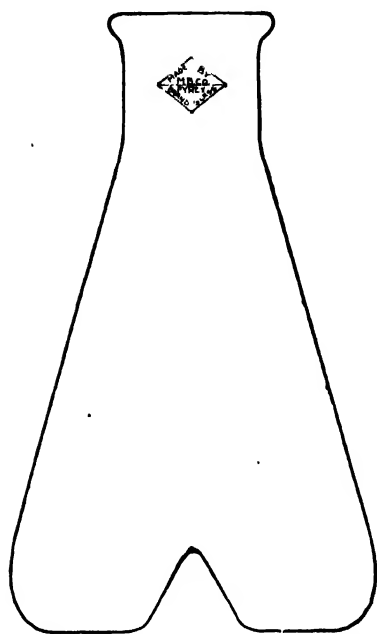


FIG. 306.—SPECIAL FLASK FOR DILUTING  
ANTIGEN FOR THE HINTON TEST

**Cholesterinized Heart Extract.**—Extract dried, ground, beef-heart muscle (Bacto-Beef Heart, Dehydrated, Difco Laboratories) by putting 100 gms. of the powder and 400 cc. of ether (anesthesia) in a wide-mouthed glass-stoppered bottle and shaking thoroughly by hand for 10 minutes. Allow the bottle to stand 5 to 10 minutes so that the solid material may settle out. Then pour as much of the ether as possible through filter paper into an Erlenmeyer flask, without pouring out any large quantity of the solid material. Scrape the solid material from the filter paper into the bottle for further extractions. Do not allow the main portion of extracted tissue to dry between extractions. Discard the filtrate in the Erlenmeyer flask. Make a total of 5 separate extractions using 400 cc. of fresh ether and a new filter paper for each. After the final extraction, let the residue dry on the filter paper. Obtain the net weight

of this dried residue of ether-insoluble constituents. Place in a glass-stoppered bottle with 95 per cent ethyl alcohol in the proportion of 5 cc. of the alcohol to each gram of residue. Extract for 3 days at room temperature ( $17^{\circ}$  to  $20^{\circ}$  C.), shaking the contents of the bottle vigorously by hand for 5 minutes 3 times each day. Remove the tissue by filtering into a graduated cylinder; measure the alcoholic extract, and transfer to a glass-stoppered bottle. Add cholesterol in the proportion of 0.4 gm. to each 100 cc., and warm at  $37^{\circ}$  C. in an incubator or water bath, occasionally shaking, until the cholesterol has dissolved.

The indicator should not be stored in a refrigerator, for chilling will precipitate the cholesterol; if, by inadvertent chilling, the cholesterol should precipitate, it must be redissolved in the same manner as that just described. Cholesterinized heart extracts kept in colorless, glass-stoppered bottles at room temperature, for a period of more than 2 years have given as good results as those freshly prepared. Samples of cholesterol obtained from Merck and from the Difco Laboratories were equally satisfactory. All of the many cholesterinized heart extracts prepared in accordance with these directions have given almost identical results.

**Five Per Cent Salt Solution.**—Prepare a 5 per cent solution of sodium chloride (C.P.) in sterile distilled water, and add 1.0 gm. of salicylic acid (C.P.) to each 4500 cc.

The salicylic acid helps to preserve the potency of glycerinated indicator (described later).

**Fifty Per Cent Solution of Glycerol.**—Prepare by mixing equal volumes of Baker and Adamson's Glycerin (Reagent) and sterile distilled water.

The 5 per cent salt solution and the 50 per cent glycerol solution keep indefinitely.

**Preparation of Glycerinated Indicator.**—This requires strict adherence to the directions for mixing. Thirty cubic centimeters is the smallest and 150 cc. the largest amount that can be satisfactorily prepared at one time. If larger quantities are required, two or more batches should be pooled. It will remain unimpaired in strength 1 month and sometimes longer if kept in a refrigerator at a temperature of about  $8^{\circ}$  C.

The glycerinated indicator is prepared as follows: Pipet 1 part of the cholesterinized heart extract into one compartment of the Erlenmeyer flask (with the inverted V-shaped ridge), and 0.8 part of the 5 per cent salt solution into the other.

Great care should be used to avoid admixture of the two solutions when the salt solution is pipetted into the flask. A 125 cc. flask is suitable for the preparation of 30 to 60 cc. of glycerinated indicator, and a 250 cc. flask for 90 to 150 cc.

Mix by shaking the flask very rapidly from side to side for 1 minute. Let the mixture stand exactly 5 minutes. Without further delay add 13.2 parts of the 5 per cent salt solution and shake thoroughly. Finally, add 15 parts of the 50 per cent glycerol solution and shake until the suspension is homogeneous.

**Procedure.**—1. Centrifuge the blood, if necessary, to aid in separating the serum from its clot, and with a long, dropping pipet remove the serum (free from blood cells) and deliver into an appropriately labelled serum tube.

To avoid contamination of one serum by another, after each has been drawn off, the dropping pipet should be thoroughly rinsed at least 3 times with sterilized physiologic salt solution; and to minimize bacterial contamination, after every 20 sera have been drawn off, the washing bottle (about 200 cc. capacity) should be emptied and filled with fresh salt solution.

2. Heat the sera in the inactivating bath at 55° C. for 30 minutes. Be sure that the level of the water in the bath is above the level of every serum and that the temperature is kept at 55° C. or 56° C. throughout the period of inactivation. Errors may result if this varies even 1 or 2 degrees. Sera should be inactivated the day of testing. If it is necessary to retest a specimen, use serum freshly separated from the clot.

3. Select all serums that show (a) hemolysis, as manifested by redness greater than that produced by dissolving 0.1 cc. of blood in 3.0 cc. of distilled water; (b) bacterial contamination, as shown by cloudiness; or (c) marked opacity from other causes. Place in a separate rack and test, as soon as conveniently possible, according to the quick method (page 723). This is done to avoid further deterioration, which decreases the sensitiveness of the test and makes it harder to read.

4. Set up the racks with one properly numbered serum tube for each of the remaining specimens. Tubes should be clear and clean. To clean the tubes, rinse them thoroughly, as soon after use as possible, with tap water, and then fill each with a warm solution of 5 gms. of sodium hydroxide in 1000 cc. of tap water; allow them to stay in this solution for about 2 hours and wash thoroughly with hot water to remove the alkali. This process usually removes (without the aid of a test-tube brush) any deposit which may have remained from previous use.

5. With a 1.0 cc. pipet, measure 0.5 cc. of each heated serum into the tube that has been labelled for it. Use a separate pipet for each serum.

For routine purposes 1 tube for each test is sufficient. If, however, this test is negative and there is reason to suspect syphilis it is desirable to retest the specimen using 0.1 cc. in one tube and 0.5 cc. in the other. In approximately one syphilitic out of 200, the tube containing the 0.1 cc. of serum gives a positive reaction, while the one which contains the 0.5 cc. of serum gives a negative reaction.

Positive and negative controls should be used if only a few tests are to be made at one time.

6. Compare the appearance of each of the pipetted serums with that in the tube from which it was taken. This is to make sure that there has been no error in pipetting or in labeling the tubes.

7. Not more than 30 minutes before incubation, with a clean 10 cc. pipet, add 0.5 cc. of the glycerinated indicator to each serum.

8. Pipet 0.5 cc. of the same indicator and 0.5 cc. of the 5 per cent salt solution into an empty serum tube. This serves as a control, the use of which will be indicated later.

9. Incline the rack to an angle of about 45 degrees and then shake by thrusting it quickly upward and forward, then downward and backward with sufficient speed to cause the fluid to travel halfway up the tubes and make small bubbles. At least 3 minutes of shaking are required for accurate results. If there are enough tests a shaking machine is desirable.

10. Place the rack in the Wassermann bath or incubator at 37° C. and let it remain for 16 hours (conveniently from 5 P.M. to 9 A.M.), or in the incubator for 18 hours. Longer incubation makes the tests increasingly hard to read. Do not agitate the contents of the tubes before reading.

When the racks containing the tests are removed from the bath or incubator, record the readings shown by the bath or incubator thermometer as well as those

of the maximum and minimum thermometer. For dependable results the temperature should not fall below 34° C. nor rise above 39° C.

**Reading the Tests.**—The tests are easier to read within an hour after the incubation than later. To read them, sit in front of a window, but do not face the sunlight. The light must be good, and for this reason suitable artificial light must be provided on dark days or at night. In order to determine whether or not there is clearing of the fluid and a ring or band of white flakes or white coarse granules at the meniscus, lift each tube carefully from the rack, hold it at the level of the eye, slant it at an angle of about 45 degrees, and view it in the direction of a darkened background on either side of a window or of a suitably placed light. While still viewing it at the same angle, slowly rotate the tube by rolling it between the fingers; this will make even a faint ring visible. Finally, gently shake the tube and look for a precipitate which may manifest itself by agglutinated masses or by only very faint granularity.

The reactions are read and reported as positive (without indicating the intensity of the reaction), negative, doubtful and unsatisfactory.

The simplicity of this method of reporting has the advantage of not confusing physicians by implying that the test is in any way a quantitative reaction, for the intensity of the reaction actually bears no relation to the clinical condition of the patient.

**Positive Tests (Recorded +).**—At or a little above the level of the meniscus there is a ring or band, approximately 0.2 to 1.5 mm. wide, of white coarse granules or flakes of lipoids slightly to moderately, but not strongly adherent to the wall of the tube. The gentle shaking loosens the ring or band and scatters the particles so that they are visible as agglutinated masses in a clear fluid or as somewhat coarse granules in a clouded fluid.

**Negative Tests (Recorded —).**—There is at most only slight clearing, but no ring, band or precipitate.

**Doubtful Tests (Recorded ±).**—Centrifuge for 10 minutes at high speed (about 2000 revolutions a minute) (1) those tubes which on gentle shaking showed only slight granularity beyond that observed in the control tube (containing 0.5 cc. of indicator and 0.5 cc. of the 5 per cent salt solution, and (2) those tubes which showed only a slightly flakey or slightly granular ring. If, as a result of the centrifuging, there is definite clearing, and on top a thin layer of lipoids which on shaking breaks up into fine flakes or coarse granules, the test is reported as doubtful and recorded ±; if these changes have not taken place, it is reported as negative.

**Quick Method.**—Treat the tests selected under No. 3 as indicated under directions 4, 5 and 6. Then add 0.5 cc. of the glycerinated indicator to each, and shake according to direction 9; next, centrifuge at 1500 to 2000 revolutions a minute for 10 minutes, read, and record as follows:

The test is positive (recorded +) if there are plainly visible flakes at the top of the fluid, and a well-marked precipitate is seen on shaking. Hemolyzed and bacterially contaminated specimens are recorded and reported as unsatisfactory unless the reaction is strongly positive. This interpretation is necessary because even the moderately hemolyzed or bacterially contaminated serum of a known syphilitic usually gives a negative reaction. In the absence of hemolysis or bacterial contamination the test is negative (recorded —) if the centrifuging has caused no change.

Whenever there is immediate need for a report, this quick method may be used



for it will detect about 80 per cent of the positive reactions obtainable by the regular test. Tubes that show only a very fine precipitate or no precipitate at all after the centrifuging should be well shaken and placed in the water bath for 16 hours, or in the incubator for 18 hours, after which the reading and interpretation are made as if the tests had been conducted in the routine manner.

### KAHN MACROSCOPIC FLOCCULATION TESTS FOR SYPHILIS

**Apparatus.**—1. *Test tubes*: These are the standard Kahn tubes, measuring 7.5 cm. in length and 1 cm. inside diameter.

2. *Antigen suspension vials*: 5.5 cm. long with an inside diameter of 1.5 cm., walls straight with flat bottoms.

3. *Pipets*: (a) 10 cc. graduated to 0.1 cc.; (b) 1.0 cc. graduated to 0.01 cc.; (c) 0.6 cc. graduated to 0.15 cc.; (d) 0.5 cc. graduated to 0.025 cc.; (e) 0.25 cc. graduated to 0.0125 cc.; (f) 0.2 cc. graduated to 0.001 cc.

4. *Test tube racks*: These are made of sheet copper, bakelite, etc. They are 11.5 inches long, 3 inches wide, and 2.75 inches high. They are constructed with 3 shelves, the upper and middle ones with 3 rows of 10 holes, each 0.5 inches in diameter. The center row of holes is offset  $\frac{1}{2}$  inch from the front and back rows.

5. *Shaking Apparatus*: The standard Kahn shaker has a speed of 275 to 285 oscillations per minute with a stroke of 1.5 inches (Fig. 307).

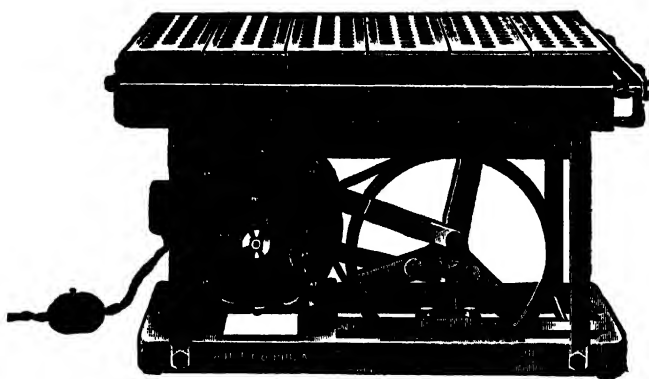


FIG. 307.—KAHN SHAKING APPARATUS

**Preparation of Reagents.**—*Serum*.—Collect blood as for the complement fixation test; separate the sera and heat at 56° C. for 30 minutes.

*Saline Solution*.—Dissolve 9 grams of chemically pure sodium chloride in 1000 cc. of distilled water and filter.

*Antigen*.—The antigen for the Kahn test is a specially prepared alcoholic extract (cholesterolized) of powdered beef heart from which the ether-soluble elements have been partially removed.

Powdered beef heart for preparing antigen is now obtainable on the market in the form of Bacto-Beef Heart, which gives highly uniform results, due undoubtedly to the fact that a large number of hearts are used in making a given lot. Because of

this uniformity, and also because of the labor- and time-saving factors, it is advantageous to employ this product in the preparation of antigen.

1. Fifty grams of powdered beef heart are placed in a 500 cc. Erlenmeyer flask. Two hundred cc. of ether (anesthesia) are added and the flask is shaken at frequent intervals for 10 minutes. At the end of this period the ether is filtered off. Gentle pressure is applied to the beef heart in the funnel by means of a spatula, to assure as complete removal of the ether as possible. The filtration is completed when practically no drops of ether pass through the funnel as a result of pressure with the spatula.

2. The moist beef heart is transferred to the original flask. This may be done by first transferring the beef heart from the funnel to a sheet of white paper and breaking the material with a spatula into pieces small enough for the mouth of the flask. One hundred and fifty cc. of ether are added to the flask, which is again shaken at frequent intervals during a 10-minute period. The ether is then filtered off as in the previous case.

3. The heart muscle is returned to the flask a third time and again covered with 150 cc. of ether. The flask is shaken from time to time during a 10-minute period and filtration carried out as previously.

4. The moist powder is then transferred to the Erlenmeyer flask for the fourth and last ether extraction. One hundred and fifty cc. of ether are added to the flask and after a 10-minute extraction period with frequent shaking, the final filtration of the ether is carried out. It is well to employ fresh filter paper for each filtration, but care should be taken to minimize the loss of powdered muscle by scraping as much of the residue as possible from the paper into the extraction flask. When the moist heart muscle has been freed from ether as completely as in the earlier filtrations, it is spread upon a sheet of white paper or a clean glass plate and dried for about 30 minutes, at room temperature. When the material is dry and free from ether odor, it is ready for extraction with alcohol. The same Erlenmeyer flask in which the ether extraction was carried out may be employed for the alcohol extraction, provided the ether has been completely removed before the adding of the powdered muscle and alcohol.

5. After completing the ether extractions, the dried powder is weighed and transferred to a 500 cc. Erlenmeyer flask. Five cc. of 95 per cent alcohol are added per gram of powder, the flask is shaken for 10 minutes, and extraction continued for 3 days at room temperature about 21° C. The flask is not shaken again during this extraction period except for a 5-minute period just before filtration. The alcoholic extract after filtration is kept at room temperature in the dark as stock solution. All corks employed in connection with the preparation and storing of antigen are covered with high-grade thin tin foil. Otherwise Vinylite in screw caps should be employed.

6. A given amount of the alcoholic extract is measured into an Erlenmeyer flask and 6 milligrams of cholesterol are added per cc. of extract. The flask is placed in a warm water bath and rotated to hasten solution of the cholesterol. When the latter is entirely dissolved, the antigen is filtered and is ready for standardization.

**Titration of Antigen.**—1. Measure 0.8, 1.0, 1.1, 1.2, 1.3 cc. respectively of 0.9 per cent salt solution into 5 standard antigen suspension vials (5.5 centimeters length, 1.5 centimeters diameter).

2. Measure into each of 5 similar vials 1 cc. of cholesterolized antigen.

3. Prepare 5 antigen suspensions by mixing the 1 cc. quantities of antigen with the varying amounts of salt solution, in series. Empty the salt solution into the

antigen and as rapidly as possible (without waiting to drain the tube) pour the mixture back and forth 6 times. Permit the mixture to stand for 10 and not over 30 minutes.

4. Test for the dispersability in salt solution of the lipid aggregate present in the antigen-salt solution suspensions after thoroughly mixing as follows:

(a) Set up 5 series of 3 standard tubes (employed in performing the regular Kahn test with serum, 7.5 centimeters length, 1 centimeter diameter).

(b) Pipet 0.05, 0.025, and 0.0125 cc. quantities of each of the 5 antigen suspensions, in series, to the bottom of the tubes, using a 0.2 or 0.25 cc. pipet marked in 0.001 or 0.0125 cc. amounts.

When measuring the antigen suspensions in series, it is advisable to begin with the suspension containing the largest amount of salt solution, and end with the one containing the least amount of salt solution. This will avoid carrying nondispersable lipid aggregates from one suspension to the other.

(c) Add 0.15 cc. salt solution to each of the 15 tubes.

(d) Shake the rack of tubes for 3 minutes in a shaking apparatus at a speed of 275 to 285 oscillations per minute. If no such apparatus is available, rapid shaking by hand will approximate this speed. The serum-antigen mixtures should stand for about 5 minutes (preferably not less than 3 minutes and not more than 7 minutes) at room temperature before the mechanical shaking for 3 minutes.

(e) Add 1 cc. salt solution to the tubes containing the 0.05 cc. amounts of antigen suspension, and 0.5 cc. to the remaining tubes. Observe whether fluids are opalescent or contain aggregates.

(f) When each of the 5 antigen suspensions are thus tested for the dispersability of aggregates, it may be found that the antigen suspensions prepared by mixing antigen with the smallest amounts of salt solution contain aggregates which are not completely dispersed in additional salt solution. The titer of the antigen is the smallest amount of salt solution which, when added to 1 cc. antigen, produces aggregates capable of complete dispersion upon the addition of further salt solution and giving an opalescent medium which is free from cholesterol crystals. If 1.3 cc. salt solution added to 1 cc. antigen results in a suspension containing particles that are not dispersed in additional salt solution, the titration is continued with volumes greater than 1.3 cc. A titer higher than 1 + 1.5 can usually be avoided by a modification of the antigen itself.

**Determination of Sensitiveness of Antigen.**—The sensitiveness of a new antigen is determined by comparing it with "standard Kahn antigen." The degree of sensitiveness of "standard Kahn antigen" was established to give as high a percentage of specific positive reactions as was possible without giving nonspecific reactions. Each new antigen that is brought to that established degree of sensitiveness becomes a "standard Kahn antigen."

1. *Preparation of Syphilitic Sera for Comparative Tests.*—Ten sera are obtained, 8 from syphilitic patients and 2 from nonsyphilitic individuals. Of the 8 sera, at least 6 should give weakly positive reactions, and the remaining, strongly positive reactions. Pooled sera may be employed. All sera are heated for 30 minutes at 56° C. before being tested. If the sera employed have been previously heated for 30 minutes, they should be reheated for 10 minutes at the same temperature before use.

2. *Testing Newly Prepared and Standard Antigen with Sera.*—Antigen suspen-

sions are prepared with both antigens in accordance with their respective titers. Both suspensions are permitted to stand for 10 minutes and each is pipetted in 0.05, 0.025, and 0.0125 cc. amounts for a series of 10 Kahn tests. The sera are then added in 0.15 cc. amounts. All the tests are shaken for 3 minutes at 275 oscillations per minute and after adding the proper amounts of salt solution to each tube, the results with the 2 antigens are compared.

3. *Interpretation of Results.*—If the results of the comparative tests with the 2 antigens are closely comparable, the new antigen probably possesses standard sensitiveness. To eliminate the possibility of error, at least 2 additional series of comparative tests are carried out, and if the results are again comparable the newly prepared antigen may be considered as standard, although it is desirable to make as many comparative tests as possible with nonpooled sera before declaring an antigen standard and satisfactory.

**Correction of Antigen.**—The sensitiveness of a newly prepared antigen may be greater or less than that of standard antigen. In either case it can readily be corrected to standard requirements. Two reagents are necessary for antigen correction: cholesterolized alcohol and sensitizing reagent.

*Preparation of Cholesterolized Alcohol.*—Cholesterolized alcohol is prepared similarly to cholesterolized antigen. Thus, for cholesterolizing 100 cc. of 95 per cent alcohol the alcohol is added to 600 milligrams of cholesterol in a 250 cc. Erlenmeyer flask or similar container. The cork to be employed should be covered with thin, high-grade tin foil. Rotate flask in a warm water bath until all cholesterol is dissolved. Filter to remove traces of foreign material. The solution is then ready for use.

*Preparation of Sensitizing Reagent.*—1. The ether filtrate obtained in the preparation of antigen from 50 grams of heart muscle is refiltered to remove traces of powdered muscle, and is then evaporated with the aid of an electric fan.

2. When the volume has been reduced to about 10 cc. or less, the concentrated ether extract is transferred to a small, weighed evaporating dish (capacity about 25 cc.), the transfer being made complete by washing out the residue into the small dish with a little ether.

3. Evaporation is continued with the aid of the fan until the ether odor is no longer detectable.

4. At this stage there may separate from the dark brown lipid mass, a few cc. of water. This water, which will be at the bottom of the evaporating dish, is removed by means of a capillary pipet. The lipid residue is brownish, semitransparent and viscous.

5. The evaporating dish is now reweighed, and the weight of the residue determined.

6. The residue is transferred to an Erlenmeyer flask (about 100 cc. capacity). This is best accomplished with the aid of a small spatula.

7. A volume of absolute alcohol equivalent to a 10 cc. per gram of residue is added to the flask. A small amount of this alcohol is employed for rinsing the evaporating dish.

8. Extraction is allowed to take place for 30 minutes at room temperature with frequent shaking of the flask.

9. The mixture is filtered, and the filtrate is allowed to stand at room temperature for 3 days. If a precipitate forms during this period, the solution is refiltered.

10. The filtrate is cholesterolized with 6 mgm. cholesterol per cubic centimeter according to the usual technic.

11. The cholesterolized extract known as "sensitizing reagent" is filtered and is ready for use.

*Methods of Correction.*—In order to understand the methods of correcting antigen to "standard" sensitiveness, it is necessary to recall that antigen sensitiveness (according to Kahn) is directly related to the concentration of lipids in the antigen. Only at a certain lipid concentration does an antigen give maximum sensitiveness, while excessive or deficient concentration reduces antigen sensitiveness. Furthermore, the degree of sensitiveness of standard Kahn antigen does not represent the maximum sensitiveness of which an antigen is capable, but instead represents a definitely chosen conservative degree of sensitiveness in conformity with specificity. Some newly prepared antigens will thus be more and some less sensitive than standard antigen, depending on their concentration of antigenic lipids.

**ANTIGENS MORE SENSITIVE THAN STANDARD ANTIGEN.**—When an antigen is more sensitive than standard antigen, it could be corrected either by concentrating the lipids of the antigen or by diluting the antigen with cholesterolized alcohol (since excessive concentration or dilution reduces antigen sensitiveness).

For simplicity, the method of choice is that of dilution. Technic: To a small amount of the oversensitive antigen, such as 10 cc., is added 1.5 cc. cholesterolized alcohol (15 per cent dilution). The diluted antigen is now tested against the standard antigen, using weakly positive sera. If comparable, the entire lot of new antigen is diluted with 15 per cent of cholesterolized alcohol. If not comparable, then if the antigen after 15 per cent dilution is still more sensitive than standard, a higher dilution, such as 25 per cent, is tried; if 15 per cent dilution reduced the sensitiveness below that of standard, a lesser dilution, such as 10 per cent, is tried.

The method of lipid concentration for reducing antigen sensitiveness is resorted to only when it is found that an antigen requires an excessive dilution (beyond 25 per cent) of cholesterolized alcohol to bring the sensitiveness to standard requirements. Technic: 1.5 cc. of noncholesterolized antigen is placed in a small evaporating dish and evaporated to dryness by means of an electric fan. The lipid residue is taken up in 10 cc. of the oversensitive antigen (15 per cent concentration). The concentrated antigen is now tested against the standard antigen as above. If the new antigen is still more sensitive than standard, a higher concentration, such as 25 per cent, is tried; if less sensitive than standard, the concentration is reduced to perhaps 10 per cent.

**ANTIGEN LESS SENSITIVE THAN STANDARD ANTIGEN.**—An antigen is less sensitive than standard when it is either too rich or too poor in lipids. An antigen which is less sensitive than standard because of excessive lipid concentration is corrected by dilution with cholesterolized alcohol, employing the identical technic described above for correcting oversensitive antigens. An antigen less sensitive than standard due to insufficient lipid concentration is corrected by adding a small amount of sensitizing reagent, such as 0.5 per cent. Technic: To 10 cc. of the less sensitive antigen is added 0.05 cc. of sensitizing reagent. The modified antigen is now compared with standard antigen in the usual way. If still less sensitive than standard, the amount of sensitizing reagent is increased to 1 or more per cent; if more sensitive, the amount of reagent is reduced below 0.5 per cent.

**VARYING THE AMOUNT OF SALT SOLUTION IN THE TITER.**—An antigen is most

sensitive when mixed with salt solution according to its titer. If the titer of an antigen, let us say, is  $1 + 1.1$ , then antigen sensitiveness is gradually reduced by employing antigen suspensions of  $1 + 1.2$ ,  $1 + 1.3$ , etc. Therefore, an antigen having a titer of  $1 + 1.1$  and more sensitive than standard antigen could obviously be brought down to standard requirements by employing titers of  $1 + 1.3$ ,  $1 + 1.5$  or even greater amounts of salt solution. In doing this, however, care must be taken not to reduce the opalescence of the completed reactions to the point where difficulty is encountered in reading results. Since most studies on the Kahn reaction have been carried out with antigen titers ranging from  $1 + 1$  to  $1 + 1.3$ , Kahn does not recommend the use of larger amounts of salt solution in the titer, and prefers reducing the sensitiveness of antigens to standard requirements by modifying the lipoidal content of the antigen.

The information gained from the standardization of the small sample of antigen may be utilized in the standardization of the entire amount of it, after which comparative tests with at least 40 sera should be made. If the comparison is favorable, the new antigen may be considered as of standard sensitiveness.

**Standard Serum Test.**—It is well to have the necessary equipment for the test ready before preparing the antigen suspension. Have racks set up, tubes numbered, sera heated and pipets ready for measuring the antigen suspension and serum. For measuring the 0.05 cc. quantities of antigen suspension, a 0.5, 1.0 or 1.5 cc. pipet may be employed, graduated in 0.05 cc. amounts. For measuring the 0.025 or 0.0125 cc. quantities, a 0.2 or 0.25 cc. pipet may be employed which is graduated either in 0.01 or 0.0125 cc. amounts. If the graduations on these pipets are not well defined, it is well to mark off the desired measurements with a wax pencil. It is essential that the temperature of the laboratory be close to  $21^{\circ}\text{C}$ .

1. *Preparation of Standard Antigen Suspension.*—Mix antigen with physiological salt solution according to required titer. Thus, if the titer is 1 cc. antigen plus 1.1 cc. salt solution, proceed as follows:

(a) Measure 1.1 cc. salt solution into a standard antigen suspension vial.

(b) Measure 1 cc. antigen into a similar vial.

(c) Pour the salt solution into the antigen, and as rapidly as possible (without waiting to drain the vial) pour the mixture back and forth 6 times to insure thorough mixing.

(d) Allow the antigen suspension to stand for 10 minutes before using. The suspension should not be used after 30 minutes standing.

One may mix more than 1 cc. of antigen with a proportionately larger amount of salt solution, but not much less than 1 cc. One cc. when mixed with salt solution will be sufficient for about 15 tests: 2 cc. of antigen mixed with salt solution will be sufficient for about 35 tests.

2. *Antigen Controls.*—After the antigen suspension has stood 10 minutes, measure 0.025 cc. into each of 3 tubes (controls) adding 0.15 cc. saline to one, 0.15 cc. negative serum to another and 0.15 cc. positive serum to the third: shake for 3 minutes, add 0.5 cc. saline to each and examine. The tubes containing positive and negative sera are controls for the sensitiveness of that particular antigen suspension. The saline control is a gage of the opalescence of the suspension, and should contain no precipitate.

3. *Measuring Antigen Suspension.*—After the antigen control tests have been

completed, shake the antigen suspension well (closing the mouth of the vial with the thumb) and measure 0.05, 0.025 and 0.0125 cc. amounts for each serum, delivering the suspension to the bottom of the tubes. When employing the standard rack which contains 30 tubes, measure 0.05 cc. amounts in the tubes of the first row; 0.025 cc. amounts in the tubes of the second row and 0.0125 cc. amounts in the tubes of the third row.

4. *Measuring Serum.*—The serum should be added as soon as possible after the antigen suspension has been pipetted, to avoid undue evaporation from the suspension. When examining large numbers of sera, it is well for one worker to measure the antigen suspension and for another to follow with the sera. Add 0.15 cc. serum to each of the 0.05, 0.025 and 0.0125 cc. amounts of antigen suspension, and shake the rack of tubes vigorously for about 10 seconds (by hand) to insure thorough mixing of the ingredients. The rack may now be set aside until a given number of tests—up to about 60—is ready for the regular 3-minute shaking period. When examining a small number of sera, it is well to permit the serum-antigen mixtures to stand for about 5 minutes (preferably not less than 3 minutes and not more than 7 minutes) at room temperature before shaking for 3 minutes. This step will render more uniform the examination of small and large numbers of specimens.

5. *Shaking.*—The standard shaking period is 3 minutes. It is important not merely to agitate the rack of tubes but to see that the fluid within the tubes is vigorously agitated. When the tests are shaken by hand, one may shake each rack for 3 one-minute periods with short rest periods. When a shaking apparatus is employed, its speed should be not less than 275 oscillations and not more than 285 oscillations per minute, with a stroke of  $1\frac{1}{2}$  inch. When shaking by hand, this speed should be approximated.

6. *Addition of Salt Solution.*—After the serum-suspension mixtures have been shaken, add 1 cc. salt solution to each tube of the first row of the rack (containing the 0.05 cc. amounts of antigen suspension) and 0.5 cc. salt solution to the remaining tubes. Shake sufficiently to mix ingredients.

7. *Reading of Results.*—Results may be read immediately after the addition of saline, but the final report should be based upon the findings after the tests have stood at room temperature 15 minutes after the addition of saline. Optimum reading conditions in each laboratory should be determined by trial.

(a) It is well to have but one source of light coming from a single window immediately in front of the observer. It will be found satisfactory to shade the upper and lower portions of the window, narrowing the source of light to a section several feet in height. Light from any other windows near the reader should be dimmed by lowering the window shades.

(b) When holding the rack in front of the exposed section of the window, the definitely positive and the negative reactions are readily differentiated without lifting the tubes from the rack.

(c) In the case of weak reactions, examine each tube individually, lifting it several inches above the eye level and slanting it until the fluid is spread into a thin layer. The precipitate will then become readily visible.

(d) Those preferring magnification will find the substage mirror of the microscope helpful. Place mirror on reading table with concave surface upward. Hold the tube in slanting position 2 or 3 inches above the mirror and examine the image in

the mirror. Either daylight or artificial light may be employed. One may also use an ordinary hand lens for reading the tests. A two- or three-fold magnification will be found satisfactory. Some workers prefer the use of a slit-light arrangement, the source of light being an electric bulb enclosed in a box which is provided with a narrow slit.

As far as possible, workers should limit themselves to one method of reading. The occasional use of magnification by readers who usually do not resort to it will be likely to affect the uniformity of their reading scale. It should be emphasized that certain highly magnifying agglutinoscopes show particles in serum alone, and are thus unfit for use in the test. The magnification must be sufficiently low as to assure opalescent and clear-cut negative reactions, with entire freedom from visible particles.

8. *Types of Reactions.*—The reactions are read on a plus-sign basis (Fig. 308).



FIG. 308.—TYPES OF REACTIONS IN KAHN TEST

(From *Kahn Test*, The Williams and Wilkins Co., Baltimore.)

(a) Four-plus reactions. In these reactions, definitely visible particles are suspended in a transparent or opalescent medium. The individual particles are readily visible by direct examination, without lifting the tubes from the rack.

(b) Three-plus reactions. In these reactions, the particles are also definitely visible, but are less clear-cut than in 4-plus reactions. The particles are not always distinguishable until the tube is lifted from the rack and examined individually.

(c) Two-plus reactions. In these reactions, finer particles are suspended frequently in a somewhat turbid medium. The particles cannot be distinguished until the tube is examined individually, usually by slanting.

(d) One-plus reactions. In these, still finer particles are suspended in a somewhat turbid medium.

(e) Doubtful reactions. In these, extremely fine particles, just within the visible range, are suspended in a somewhat turbid medium.



(f) Negative reactions. In these, the medium is transparent and opalescent and free from visible particles. In the rack, negative reactions are readily distinguished from weakly positive reactions by the fact that the latter appear turbid.

The American Committee on Evaluation of Serodiagnostic Tests for Syphilis recommends that reports be rendered as positive, doubtful or negative.

9. *Interpretation of Results.*—(a) Each tube is read and recorded individually. A definite precipitate, the particles of which are surrounded by a clear medium, is read as 4 plus. Proportionately weaker reactions are read as 3 plus, 2 plus, 1 plus, or doubtful, respectively. An absence of any precipitate is read as negative.

(b) Specimens of serum that are not strongly potent will not show complete precipitation in all 3 tubes. The strongest reactions will be seen in the back tube and the weakest in the front tube, the reason being that the small amount of reagin (antibody) in the serum reacts better with the smaller amount of antigen suspension. Such sera usually show weak reactions in the middle tube with its moderate amount of antigen suspension and faint to no precipitate in the front tube which has the largest amount of antigen suspension.

(c) Strongly potent serum specimens will show a 4-plus reaction in each tube, but due to the different amounts of antigen suspension used in each, the precipitates will be unequal in bulk, being greater in the front and least in the back tube.

(d) Sera with very high potency often produce a so-called reversed reaction in which the front tube is markedly positive, or at least definitely positive, while the middle and back tubes are relatively weaker. In these cases the large amount of reaction reagin present in the specimen requires a large amount of antigen suspension to produce complete precipitation.

(e) Supplementary tests employing larger amounts of antigen suspension in relation to the amount of serum is necessary to correctly differentiate such specimens. The following supplementary tests must be set up in these cases.

No. 1.—In this test serum-antigen suspension ratio of 2—1 and 1—1 are used.

	Tube No. 1	Tube No. 2
Antigen suspension cc. ....	0.025	0.025
Serum cc. ....	0.025	0.05
Shake for 3 minutes		
Salt solution cc. ....	0.3	0.3

When a precipitate occurs in both tubes, or at least in tube No. 2, the reaction is reported as *strongly positive*.

No. 2.—In this test the serum is diluted 1—5, 1—10, and 1—20 with salt solution and each dilution tested with antigen suspension in a proportion of 15—1, in accordance with the following outline:

	Tube No. 1	Tube No. 2	Tube No. 3
Antigen suspension cc. ....	0.01	0.01	0.01
Diluted serum cc. ....	0.15	0.15	0.15
(Dilution) ....	(1—5)	(1—10)	(1—20)
Shake for 3 minutes			
Salt solution cc. ....	0.5	0.5	0.5

When a precipitate occurs in one or more tubes the test is *positive*.

If both these supplementary tests are negative the standard test which showed a reversed reaction should be reported as a *weak* or *doubtful* reaction.

(f) Occasionally one sees border-line reactions in each of the 3 tubes, such as, plus-minus, plus-minus, plus-minus or 1 plus; 1 plus, 1 plus or even 2 plus, 2 plus, or 2 plus. When this occurs examine the serum to see that it is clear. If it is clear carry out the above supplementary tests and if they show a definite precipitation report as positive. If the supplementary tests show border-line or questionable reactions report as weak or doubtful because, it is probable that one may be dealing with a nonspecific reaction.

(g) The following types of reaction are reported and recorded as *positive* (++++):

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	++++	++++	++++
2.	+++	++++	++++
3.	++++	+++	+++
4.	++++	++++	++
5.	++++	++++	+
6.	++++	++++	negative

Experience has shown that serums reacting as in 4, 5 and 6 are strongly positive. The following types of reaction are reported as *positive* (+++):

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	++	++++	++++
2.	+	++++	++++
3.	—	++++	++++
4.	++	+++	+++

(h) The following types of reaction are reported as *positive* (++++) when the supplementary tests are positive. If the supplementary tests are negative, reactions 1 and 2 are reported as *positive* (+++), reactions 3, 4, 5, and 6 are reported as *doubtful* (+), and reactions 7 and 8 are *negative*:

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	++++	+++	—
2.	++++	++	—
3.	++++	+	—
4.	++++	—	—
5.	+++	—	—
6.	++	++	++
7.	+	+	+
8.	±	±	±

(i) The following types of reaction are reported as *positive* (++):

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	—	++	++++
2.	—	+	++++
3.	—	+++	+++
4.	—	++	+++
5.	+	++	++

(j) The following types of reaction are reported as *doubtful* (+):

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	—	—	++++
2.	—	—	+++
3.	—	+	++
4.	—	++	++

(k) The following types of reactions are reported as *doubtful* ( $\pm$ ):

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	—	$\pm$	++
2.	—	—	++

(l) The following types of reaction are reported as *negative* ( $\pm$ ):

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	—	+	+
2.	—	$\pm$	+

(m) The following types of reactions are reported as *negative*:

	<i>Front Tube</i>	<i>Middle Tube</i>	<i>Back Tube</i>
1.	—	—	—
2.	—	—	+

(n) There must be no question as to the correctness of the results because of a possible technical error, or because of the characteristics of the specimen itself. If doubt does exist, repeat the test, even if it is necessary to request another specimen.

(o) Workers must avoid becoming too "sensitized" in reading results. They must not see precipitates where none exist. They must familiarize themselves, not only with the appearance of the negative controls, but with negative serum alone as well. Any of these, under sufficient magnification, will begin to show evidence of the very fine particles that are present in all colloidal solutions but which have no connection with the precipitate of a positive reaction. Scratched and scored test tubes should be avoided as much as possible because they interfere with the proper visualization of a precipitate.

10. *Procedure with Less Than Three Tubes.*—If there is insufficient serum for the regular 3-tube test, examine and report as follows:

(a) If enough serum for 2 tubes, employ the lesser amounts of antigen suspension; report as a 2-tube test.

(b) If enough for 1 tube, employ the least amount of antigen suspension; report as a 1-tube test.

(c) If less than 0.15 cc. serum is available, a 1-tube test (micro) may be made by employing 10 parts of serum to 1 part of antigen suspension. Thus, if 0.05 cc. serum is available, it is employed with 0.005 cc. antigen suspension. Report these reactions as micro tests.

**Quantitative Serum Test.**—The standard test is only partially quantitative. A quantitative relationship exists between sera giving +++++, +++++, +++, + or  $\pm$ , but 2 sera giving +++++ reactions might show marked variation in potency. The degree of this variation may readily be determined by means of the quantitative

procedure. Only positive sera are employed. The test consists of 2 steps. The sera are first diluted in series with salt solution, then each dilution is tested with antigen suspension, and the highest dilution giving a positive precipitation reaction is the end point desired.

Many workers use the more sensitive "sensitized" antigen in preference to standard antigen in the quantitative tests with serum and spinal fluid. Since these tests are made only in cases in which syphilis is definitely established, it is believed that the use of a highly sensitive method is more desirable than the use of a conservative method.

1. *Serum Dilutions*.—Prepare a series of 8 serum dilutions with 0.9 per cent salt solution, so that the ratio of the volume of diluted serum to the volume of serum before dilution ranges from 1 (undiluted serum) to 60 (1 part serum plus 59 parts salt solution). The following scheme is employed:

Dilution Number	Dilution Ratio	
(1)	1	= undiluted serum
(2)	5	= 0.2 cc. undiluted serum plus 0.8 cc. salt solution
(3)	10	= 0.7 cc. of (2) plus 0.7 cc. salt solution
(4)	20	= 0.2 cc. of (3) plus 0.2 cc. salt solution
(5)	30	= 0.2 cc. of (3) plus 0.4 cc. salt solution
(6)	40	= 0.1 cc. of (3) plus 0.3 cc. salt solution
(7)	50	= 0.1 cc. of (3) plus 0.4 cc. salt solution
(8)	60	= 0.1 cc. of (3) plus 0.5 cc. salt solution

2. *Antigen Suspension*.—Prepare standard antigen suspension as for regular 3-tube test (or sensitized antigen as described under Presumptive Procedure). Measure 0.01 cc. amounts of antigen suspension into 8 standard test tubes.

3. *Measuring Serum Dilutions*.—Add 0.15 cc. amounts of the 8 serum dilutions, in order, beginning with the highest dilution (8), to the tubes containing antigen suspension. Mix ingredients by shaking for about 10 seconds.

4. *Shaking*.—Shake the mixtures of serum and antigen suspension by hand or in shaking apparatus for 3 minutes at 275 to 285 oscillations per minute.

5. *Addition of Saline*.—Add 0.5 cc. salt solution to each tube and shake by hand for a few seconds to mix ingredients.

6. *Reading Results*.—After the salt solution has been added, the results are read. Record a definite precipitate (++++) , (+++ or ++) as positive and a weak or negative precipitate (+, ± or —) as negative.

7. *Interpretation of Results*.—(a) A definite precipitate, (4 plvs, 3 plus or 2 plus) is recorded as positive while weaker reactions are disregarded. If a precipitate (4 plus, 3 plus or 2 plus) occurs in the undiluted tube only but in none of the dilution series, the results are reported as 4 units, 3 units or 2 units respectively.

(b) When the reaction is of 2 plus or stronger in the dilution tubes, the potency is determined by the formula  $S \text{ equals } 4 D$ , in which  $S$  is the Kahn units and  $D$  is the highest dilution giving the reading. Thus, if the readings in the 8 tubes are as follows:

Tube:							
1	2	3	4	5	6	7	8
++++	++++	+++	++	+	neg.	neg.	neg.

The 4th tube, with a dilution of 1:20, is the highest dilution showing a reaction of 2 plus or greater. In this case S equals 4 D; or S equals  $4 \times 20$  or 80 units.

(c) Serums giving a 2 plus reaction in the 8th tube necessitate the carrying out of the dilution beyond the dilution ratio of 60. Higher dilutions are readily prepared by resorting to tube 3 (1-10 dilution) in the dilution series, of which an excess is prepared.

**Presumptive Serum Test.**—The presumptive test is a 1-tube procedure and is more sensitive than the regular Kahn test by virtue of the fact that it utilizes a highly sensitive antigen known as "sensitized antigen." As is true in the case of standard Kahn antigen, sensitized antigen also possesses a uniform degree of sensitiveness. In preparing this antigen, standard antigen is used as a base and is brought to the required sensitiveness of sensitized antigen by means of sensitizing reagent in combination with cholesterolized alcohol. Experience has shown that the addition of 1 to 25 per cent sensitizing reagent to standard antigen followed by the addition of 10 or 25 per cent cholesterolized alcohol will considerably increase the sensitiveness of standard antigen. This fact is generally utilized in the standardization of sensitized antigen.

**Preparation of Sensitized Antigen.**—In preparing sensitized antigen using standard antigen as a base, the following steps are employed:

1. To 10 cc. of standard antigen are added 0.1 cc. of sensitizing reagent and 1 cc. cholesterolized alcohol (1 per cent sensitizing reagent plus 10 per cent dilution with cholesterolized alcohol).

2. To a second 10 cc. amount of standard antigen are added 0.2 cc. of the sensitizing reagent and 1 cc. cholesterolized alcohol (2 per cent sensitizing reagent plus 10 per cent dilution with cholesterolized alcohol).

**Titration of Antigen.**—These two modified antigens are titrated in the usual manner to determine the smallest amount of salt solution to add to 1 cc. of antigen resulting in an antigen suspension, the aggregates of which will completely disperse in additional salt solution or in nonsyphilitic serum. In making this titration, antigen suspensions are prepared by mixing 1 cc. amounts of antigen with 1.7, 1.9, 2.0, 2.1 and 2.2 cc. quantities of salt solution, respectively. After these suspensions have stood for 30 minutes at room temperature, they are shaken and examined for the dispersability of the aggregates as follows: Each of the antigen suspensions is tested by employing the regular 3-tube test except that after depositing the 0.05, 0.025 and 0.0125 amounts of the suspension in 3 tubes, 0.15 cc. amounts of 0.9 per cent salt solution instead of serum are added to each tube. After the usual 3-minute shaking period, 1 cc. salt solution is added to the tube containing the 0.05 cc. amount of antigen suspension and 0.5 cc. amounts of salt solution to the remaining tubes. The antigen suspension containing the smallest amount of salt solution and having aggregates which are completely dispersable in the additional salt solution, represents the titer of each of the modified antigens.

**Determination of Sensitiveness of Antigen.**—1. The modified antigens at their titers are then compared in sensitiveness with a known standard sensitized antigen employing 9 weakly positive sera and 1 negative serum. In these comparative examinations the regular 3-tube test is employed. If one of the modified antigens is comparable in sensitiveness to the sensitized antigen, the comparative examination is repeated with 20 additional weakly positive and 20 negative sera, and if the results

are again comparable, the new antigen is considered as standard and satisfactory sensitized antigen.

2. If neither one of the 2 modified antigens conforms to the requirements of standard sensitized antigen, other combinations of sensitizing reagent and cholesterolized alcohol are tried.

When the correct sensitiveness has been obtained with the sample of antigen, any desired amount may be prepared, adhering to the proportions experimentally established. The new antigen should then be compared with standard sensitized antigen, using at least 40 sera. If the comparison is favorable, the new antigen may be considered ready for use.

After the desired potency of sensitized antigen has been obtained, any amount of the antigen can be prepared by adding to standard antigen the determined amounts of sensitizing reagent plus cholesterolized alcohol.

*Performance.*—1. Pipet 1 cc. of standard sensitized antigen into an antigen suspension vial.

2. Pipet an amount of 0.9 per cent salt solution, indicated by the titer, into a similar vial.

3. Pour the salt solution into the antigen and, as rapidly as possible, pour the mixture back and forth 6 times.

4. Allow the antigen suspension to stand 10 minutes at room temperature before using.

5. Prepare antigen controls as with standard antigen.

6. Measure 0.025 cc. of the thoroughly mixed antigen suspension into a standard tube (7.5 centimeters in length, 1 centimeter in diameter) with a 0.25 cc. pipet marked in 0.025 cc. amounts or with a 0.2 cc. pipet marked in 0.001 cc., delivering to the bottom of the tube.

7. Add 0.15 cc. serum, after heating for 30 minutes at 56° C., with a 1 cc. pipet graduated in 0.01 cc. and mix the serum with the antigen suspension by shaking the rack vigorously by hand for about 10 seconds.

8. Shake rack in the usual manner for 3 minutes (oscillation speed 275 to 285 per minute).

9. Add 0.5 cc. 0.9 per cent salt solution to the tube and examine for presence of precipitates.

*Reading Results.*—The results are read immediately and the tubes are examined individually with the aid of the concave side of the microscope mirror and adequate light. Those showing absolutely no reaction can be reported negative without further tests. Those showing any reaction, plus minus or greater, must be examined by the standard serum test. If these sera are examined the same day by the standard test, they are reheated for 10 minutes. If as much as 24 hours elapses before they are re-examined, they are reheated for 15 minutes.

**Standard Spinal Fluid Test.**—In this test, the greater part of the globulins is precipitated by means of ammonium sulphate and redissolved in an amount of saline solution equivalent to a tenth of the original spinal fluid volume. The concentrated globulin solution thus obtained is then tested with antigen suspension.

*Preparation of Globulin Solution.*—(a) Centrifuge spinal fluid to render it free from cells and foreign particles; (b) place 1.5 cc. of the clear fluid in a test tube; (c) add 1.5 cc. of a saturated solution of ammonium sulphate; (d) mix fluids, cover-

ing mouth of tube with thumb (protected with rubber if desired) and shake tube back and forth vigorously. The thorough mixing of the spinal fluid and ammonium sulphate is of great importance. Place mixture in 56° C. water bath for 15 minutes to hasten the precipitation of the globulins; (e) centrifuge mixture at high speed for about 15 minutes to completely throw down the precipitated globulins; (f) remove the supernatant fluid *as completely as possible*. This is best accomplished with the aid of a finely drawn capillary pipet. The major amount of supernatant fluid is first withdrawn. The tube is then slanted at an angle of about 45 degrees and the remaining fluid is withdrawn after bringing the opening of the capillary pipet to the point of contact of the globulin precipitate and the inner wall of the tube. It will be found that the last trace of supernatant fluid can be removed by this method. Some workers prefer to pour off the supernatant fluid and place the inverted tube in a rack having a layer of filter paper on the bottom. It will be found that after about 10 minutes standing, the fluid in the tube will be completely drained and absorbed by the filter paper; (g) add 0.15 cc. salt solution to the precipitate and redissolve it by gentle shaking. In adding this salt solution the point of the pipet should be lowered close to the bottom of the tube to avoid washing down the ammonium sulphate adhering to the inner wall. The globulin precipitate will dissolve readily. This globulin solution is now ready to be tested with antigen suspension.

**Procedure.**—1. Mix salt solution with antigen in the same manner as for the test with serum, according to the antigen titer required for spinal fluid. The antigen suspension should stand 10 minutes before its use in the test and should be used within 30 minutes. Control tests of the antigen suspension should be made, as outlined under "Performance of the Standard Test" for serum.

2. With a 0.2 cc. pipet graduated to 0.001 cc. measure 0.01 cc. of antigen suspension to the bottom of a standard Kahn test tube.

3. Measure 0.15 cc. of concentrated solution into the antigen suspension tube, using a 0.2 cc. pipet. Shake tests vigorously for 10 seconds to mix ingredients.

4. Include positive and negative spinal fluid controls; also observe each concentrated globulin solution to establish that it is free from foreign particles.

5. After mixing the concentrated fluid with antigen suspension, shake test at standard speed for 4 minutes. This period is more desirable for spinal fluids than 3 minutes.

6. Add 0.5 cc. 0.9 per cent salt solution to tube.

7. A definite precipitate suspended in a clear medium is read + + + +. Proportionately weaker precipitates are read + + +, + + and + respectively. Four plus, 3 plus and 2 plus reactions are reported as positive; 1 plus reactions are reported as doubtful; plus minus and negative reactions are reported as negative. Each spinal fluid examination should be performed in duplicate. If the spinal fluid contains blood it should be so noted in the report.

**Presumptive Spinal Fluid Test.**—This test is carried out essentially as the standard test with spinal fluid, except that sensitized antigen is used.

**Quantitative Spinal Fluid Test.**—Employing spinal fluids that are known to give positive reactions, a series of dilutions are made similar to those of positive sera. The dilution range is from undiluted to 1:40. Undiluted spinal fluid is considered as equivalent to a 1:10 dilution, since the standard test is performed with a solution in which the globulin is concentrated to  $\frac{1}{10}$  the original volume. In the performance of the

test, 0.01 cc. antigen suspension is mixed with 0.15 cc. of each of the spinal fluid dilutions. The mixtures are shaken for 3 minutes, 0.5 cc. salt solution is added to each tube and the results are read on a similar basis to the quantitative serum tests previously described.

### KLINE MICROSCOPIC FLOCCULATION TEST FOR SYPHILIS

**Sera.**—These are prepared as for the Wassermann test, care being exercised that they contain no red blood cells, or foreign particles. (They are heated at 56° C. for 30 minutes.)

When blood is obtainable in small quantity only it is advisable to collect this in a narrow test tube (about 8 to 9 mm.) and to handle it in the same manner as a larger sample from the vein.

When blood is obtainable in very small quantity only it is advisable to collect this in a narrow glass tube with a capillary end. The end is then sealed, a narrow rod is passed through the open end to free the clot from the wall and after the tube is centrifuged at high speed, the serum is removed and placed in a water bath at 56° C. with water above the upper level of the serum. After inactivation, the tube is filed and broken just above the clot and the serum allowed to run into or is drawn into a 1 cc. pipet, graduated in hundredths.

**Glassware.**—Microscopic slides 2 × 3 inches as purchased are rubbed on both sides with Bon Ami paste (prepared by breaking up a cake of Bon Ami in a small quantity of hot water). As soon as the paste is dry (in about 5 minutes) it is completely removed from the slide with a soft muslin cloth. For convenience the slides covered with paste may be stuck to each other, allowed to dry, and cleaned at any time.

Upon clean slides for the heated serum tests, 12 paraffin rings, each with an inside diameter of 14 mm. are mounted. Inasmuch as the slide test results are influenced by the surface area of the chambers it is important that the paraffin rings be thin ones. With a little practice the required amount of paraffin can be ascertained. At first, it may be difficult to make complete rings. These incomplete rings may be completed by applying the loop a second time to the open areas.

For the spinal fluid tests double ring slides are prepared as follows: Upon the clean slide a steel mold  $3 \frac{2}{16} \times 2 \frac{3}{16} \times \frac{1}{8}$  inches with 2 central walls  $1 \frac{0}{16}$  inches in diameter is placed. A metal disk  $1 \frac{5}{16}$  inches in diameter and  $\frac{3}{16}$  of an inch thick is then placed in the center of each well. The space between them is filled with hot wax (2 parts ordinary vaseline and 1 part parowax) from a 10 cc. glass syringe. After the mixture cools a few minutes, each disk is elevated from the slide and separated from the wax wall by turning the central screw handle a few times to the right (holding mold down at edge). After the disk is freed, it is lifted out. The mold is removed by inserting a thin blade between it and the slide.

**Pipets.**—The pipets needed for delivering sera, and spinal fluid, and those for preparing the antigen emulsions are the ordinary finely graduated 0.2 to 10 cc. pipets. The pipet for the 1 per cent acetic acid is a 0.2 cc. pipet graduated in 0.001 cc. The pipets for delivering the antigen emulsions are Wright pipets made from glass tubing 6 to 10 mm. in diameter with the tubes about 0.5 mm. in outside diameter, delivering a drop equal to about 0.008 cc. (62 drops per 0.5 cc.).



**Instrument for Making Paraffin Rings.**—This is essentially the instrument proposed by Green. A piece of soft iron wire (No. 28) is wound twice tightly about a test tube (about 15 mm. in outside diameter) forming a double loop and leaving a double shaft about an inch in length. The 2 shafts are then twisted together to within a quarter of an inch of the free end. After removing the looped wire from the test tube, a piece of linen thread (No. 12) is started from the free end of the shaft after being fastened here by a single twist of the free ends. Three long turns are made reaching the loop which is then tightly wound with the thread. The winding is continued up the shaft to the free end where it is fastened between the 2 ends of the wire by twisting them. The loop is then bent at right angles to the shaft. It is then re-shaped by working the loop against the bottom of the test tube mentioned above. The shaft is then inserted into the handle of a teasing needle or into a straight hemostatic forceps.

The paraffin rings are made by dipping the instrument into smoking paraffin (about 120° C.) draining quickly at one point and transferring the remainder to the glass slide.

**Slide Holders.**—The slide holder (for 3×2 inch slides) is a wooden lid of a slide box (3½×6¾×½ inches) containing an easily fitting thin wooden shelf having a small handle at each end.

**Salt Solution.**—0.85 per cent sodium chloride (C.P. or reagent, Merck) solution used in the tests is prepared with distilled water having a pH of about 6. (Such water gives a lilac color when 1 drop of chlorphenol red indicator (LaMotte) is added to 0.25 cc. of it in a small chamber). Distilled water having a pH of 5.2 or less gives a yellow color with this indicator and is not satisfactory.

**One Per Cent Acetic Acid (C.P. Reagent).**—It is advisable to use no less than 1 cc. of acid (delivered from a 1 or 2 cc. pipet) and accordingly 99 cc. of distilled water.

**Antigen.\***—The purified antigen used in the microscopic slide precipitation tests for syphilis is prepared as follows:

1. Two hundred grams of dried heart powder (Difco) is placed in a 2 liter Erlenmeyer flask.

2. One liter of absolute ethyl alcohol (99 + per cent) (Rossville Commercial Alcohol Corp., Lawrenceville, Ind.) is added.

3. After the flask is stoppered with a cork covered with tin foil, it is shaken vigorously by hand at intervals for 2 hours. Better still 2 wide mouth bottles (Difco bottles for 1 pound beef heart powder) each with 100 grams of beef heart powder and 500 cc. of absolute ethyl alcohol (99 + per cent) are shaken vigorously in a machine for 2 hours. (This short extraction removes almost all of the desired antigenic substance in the powder.)

4. The extract is filtered into a liter cylinder through good grade filter paper of medium texture (Schleicher and Schull No. 597, 38.5 cm.).

5. During filtration the mixture is stirred with a wooden tongue depressor and toward the end pressed with the cork until the powder is quite dry.

\* Standard materials including antigen for the microscopic slide precipitation tests for syphilis may be obtained from the LaMotte Chemical Products Company, McCormick Building, Baltimore, Md.

6. The extract (about 775 cc.) is placed in the refrigerator at 8° to 10° C. for 24 hours.

During this time a fairly heavy white precipitate settles out. This is filtered off and the filtrate in a large evaporating dish is concentrated on a water bath at 45° to 50° C. determined by a thermometer bulb within the extract. During evaporation of the alcoholic extract an irregular festoon appears at the periphery. When the extract reaches the proper concentration the festoon disappears and the margin of the concentrated extract is sharp.

7. The extract is now poured quickly into 500 cc. of acetone, C.P. (Coleman and Bell) at 50° C. in a large evaporating dish.

8. The dish is then placed in an air incubator at 37° C. for 15 minutes after which the acetone is decanted leaving a soft yellow brown wax adherent to the side of the dish. (Longer periods of precipitation and precipitation at lower temperatures permit of precipitation of adventitious substances as well and such antigens give more sensitive and less specific results.)

9. The dish is then placed on a water bath or in an air incubator at 50° C. until the little acetone remaining has evaporated (about 30 minutes).

10. The wax is then worked together and placed in a glass-stoppered bottle. Then 80 cc. of absolute ethyl alcohol (99 + per cent) that has been kept in an air incubator at 50° to 56° C. for ½ hour or longer, is added and after a few minutes' shaking the bottle is placed in an air incubator at 50° C. and shaken gently after 15 minutes and again after 30 minutes, when it is removed from the incubator and placed in the refrigerator at 8° to 10° C. for 45 minutes.

11. The solution is then filtered and the filtrate is evaporated down at 45° to 50° C. resulting in a soft brown wax (antigen wax). The wax is weighed and to each gram in a glass-stoppered bottle, 10 cc. of absolute ethyl alcohol (99 + per cent) (at 50° to 56° C.) is added. After the bottle is shaken for a few minutes it is placed in an air incubator at 50° C. for 30 minutes, and then shaken for a few minutes.

12. The slightly turbid solution is then placed at 8° to 10° C. for about an hour and then filtered. The resultant clear filtrate is the antigen, and contains about 8.75 per cent of the alcohol-treated acetone-insoluble wax.

13. The antigen, however, is purified by extraction with water (*Am. Jour. Clin. Path.*, 12: 48, 1942) as follows:

In a warm room (70° F. or more) pipet into a liter Erlenmeyer flask, with flat bottom 5 inches in diameter, 5 cc. of distilled water (pH about 6).

Pipet in, against the neck of the flask, 10 cc. of standard slide test antigen.

Rotate the flask on a flat surface for 1 minute with considerable vigor, then rotate more gently for 1 minute controlling the motion to collect the wax, which has precipitated out, toward the center. (Proper surface in the bottom of the flask and proper vigor of mixture are necessary to produce the maximum precipitation of the wax.)

Allow the flask to stand until the precipitated wax settles and sticks to the bottom (about 15 seconds).

Carefully decant the fluid by slowly tilting the flask forward on a flat surface to a level just permitting the fluid to run out.

Continue the tilting, after the wax sticks, until the flask is completely inverted.

Allow the flask to drain on a piece of gauze or filter paper for 2 to 3 minutes.

Dry the inner wall of the flask with a gauze sponge for a distance of a few inches and place it with the open end up in the air incubator at 50° C. for a few hours. At the same time, place in the incubator, an opened glass stoppered bottle into which the purified antigen is to be pipetted, a bottle of absolute ethyl alcohol and a 10 cc. pipet.

After the materials have been heated for a few hours, 5 cc. of the warm ethyl alcohol in the incubator are transferred in the warm pipet to the warm Erlenmeyer flask containing the purified antigen wax.

A paper cup is placed as a cap over the open end of the flask and after it has remained in the incubator for 15 minutes the flask is shaken inside the incubator, several times to complete the solution of the wax.

The flask is then tilted forward, in the incubator, to collect the fluid within a small space and the concentrated water purified antigen solution is pipetted off and transferred to the warm dry bottle within the incubator (about 3 cc. of the original 5 cc. will be recovered).

Again add 5 cc. of warm absolute alcohol to the flask, inside the incubator, then shake it several times and again pipet off all the fluid obtainable (about 4½ cc.) and transfer it to the antigen bottle inside the incubator.

For the third time add warm absolute alcohol to the flask in such quantity that when as much as possible of it is transferred, the total in the antigen bottle will be 10 cc.

**Preparation of Antigen Emulsions for Diagnostic and Exclusion Slide Tests of Heated Serum.—Formula.**—0.85 cc. of distilled water (pH about 6).

1.0 cc. 1 per cent cholesterin (C.P. Pfanstiehl) in absolute ethyl alcohol (99 + per cent).

0.1 cc. antigen.

2.45 cc. 0.85 per cent sodium chloride (C.P. or reagent, Merck) solution (pH about 6).

The 1 per cent cholesterin solution for the emulsions is prepared in about 45 minutes by placing the cholesterin flakes and absolute alcohol in a glass-stoppered bottle in an oven at 50° to 56° C. and shaking gently a few minutes at 15-minute intervals. The solution kept in the incubator at 37° C. is thoroughly satisfactory for use as long as 2 months.

The sensitivity of an emulsion is greatly influenced by the quantity of cholesterin present. The sensitivity is likewise influenced by the quality of the cholesterin used. The cholesterin (Pfanstiehl, C.P.) that has been found uniformly satisfactory in the slide tests is flaky, pearly and readily soluble to 1 per cent in absolute ethyl alcohol (99 + per cent). Powdery, white cholesterin incompletely soluble to 1 per cent in absolute alcohol (99 + per cent) has been found to give too sensitive results.

The technic of preparing the emulsion according to the above formula is as follows: Into a 1 ounce bottle the required amount of distilled water (pH about 6) is pipetted.

The bottle is held at an angle, and the 1 per cent cholesterin in absolute ethyl alcohol (99 + per cent) is allowed to run along the side of the neck of the bottle.

The bottle is gently rotated from the neck for 20 seconds.

It is held at an angle again, and the proper amount of antigen is pipetted against the side of the neck of the bottle from a finely graduated pipet.

The bottle is promptly stoppered with a cork and shaken vigorously (the fluid thrown from bottle to cork and back) for 1 minute.

Lastly, the 0.85 per cent sodium chloride solution is allowed to run in quite rapidly, the bottle is stoppered again and shaken less vigorously than previously for 1 minute.

The emulsion, when examined under the microscope at a magnification of about 120 times, shows numerous very fine particles, but no clumps whatever.

#### *For Diagnostic Test*

Place 1 cc. or more of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 35° C. (beaker of water in usual laboratory air incubator at about 37° C.) for 15 minutes. The emulsion as soon as heated is ready for use.

#### *For Exclusion Test*

Place 2 cc. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 56° C. for 15 minutes. Then pour into a 3 × 1 inch tube and centrifuge for 15 minutes (eighth setting Rheostat, Centrifuge Size 1, Type SB). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1.5 cc. of 0.85 per cent sodium chloride solution. Transfer to a narrow tube for use.

These emulsions, kept at room temperature, are satisfactory for use for 48 hours after preparation.

**Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Heated Serum.**—1. Place 3 heated serum test slides each with 12 small chambers, on a tray in a small holder.

2. Into each of the 36 rings, pipet 0.05 cc. of the heated serum to be tested (18 sera in duplicate).

3. After all the sera are pipetted, 1 drop of the diagnostic test antigen emulsion (about 0.008 cc.) is allowed to fall from a Wright pipet into one of the two portions of each serum. Into each of the other 18 duplicate sera a similar drop of exclusion test antigen emulsion is allowed to fall from a Wright pipet.

4. The slides in the holder are rotated on a flat surface for 4 minutes.

5. The results are examined at once through the microscope at a magnification of about 120 times (low power 16 mm. objective, eyepiece 12) with the light cut down as for the study of urinary sediments and reported in terms of pluses according to the degree of clumping and the size of the clumps (Fig. 309).

Any spilling from the chamber makes the reaction therein unsatisfactory, and the serum concerned should be retested.

If sufficient serum is available the exclusion test for syphilis may be done with 0.3 cc. heated serum in a chamber similar to that employed for the spinal fluid test (33 mm. in diameter) and 1 drop about 0.008 cc. of emulsion made by suspending the sediment of 8 cc. of exclusion test emulsion (centrifuged 15 minutes at 8th rheostat setting) in 1 cc. of 0.85 per cent salt solution.

**Preparation of Antigen Emulsions for Diagnostic and Exclusion Slide Tests of Spinal Fluid.**—*Formula.*—0.85 cc. distilled water (pH about 6).

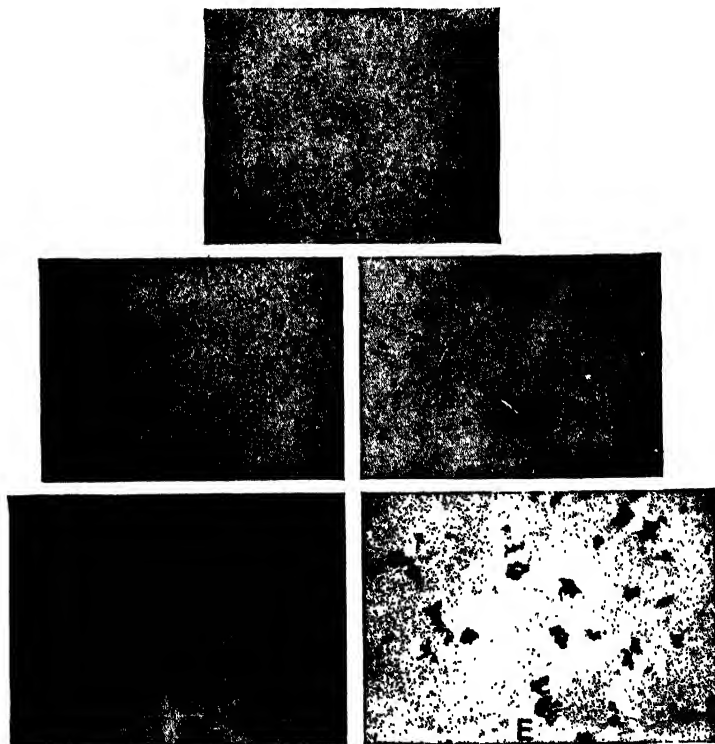


FIG. 309.—THE KLINE MICROSCOPIC PRECIPITATION REACTION

A, negative reaction; B, positive (+); C, positive (++); D, positive (+++); E, positive (++++)

1.25 cc. of 1 per cent cholesterin (Pfanstiehl C.P.) in absolute ethyl alcohol (99 + per cent).

0.1 cc. antigen.

2.2 cc. of 0.85 per cent sodium chloride (C.P. or reagent, Merck) solution (pH about 6).

8.8 cc. of the emulsion are made by using double the quantities given in the formula.

#### *For Diagnostic Test*

Place 4 cc. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 35° C. for 15 minutes. Then pour into a 3 × 1 inch tube. Centrifuge for 15 minutes (eighth setting Rheostat, size 1, S.B.) Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1 cc. of 0.85 per cent sodium chloride solution (pH about 6).

Transfer to a narrow test tube for use.

#### *For Exclusion Test*

Place 4 cc. of the emulsion in a narrow test tube (12 mm. inside diameter) in a water bath at 50° C. for 15 minutes. Then pour into a 3 × 1 inch tube. Centrifuge for 15 minutes (eighth setting Rheostat, size 1, S.B.). Decant the fluid and, with the tube inverted, dry the inside of the tube with a cloth almost to the level of the sediment. To the sediment add 1 cc. of 0.85 per cent sodium chloride solution (pH about 6).

Transfer to a narrow test tube for use.

These emulsions, kept at room temperature, are satisfactory for use for 24 hours.

**Diagnostic and Exclusion Microscopic Slide Precipitation Tests for Syphilis with Spinal Fluid.**—*Preliminary Procedures.*—Spinal fluids, turbid with exudate, blood or bacteria, or containing injected substances including horse serum, are unsatisfactory for testing. Spinal fluids with slight turbidity or few particles are centrifuged at high speed for 10 minutes, and the clear fluid is withdrawn or decanted.

Place the required number of test tubes,  $6 \times \frac{5}{8}$  inches, each containing 5 cc. of Benedict's solution (1909-1910), in a beaker (Pyrex). Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for 5 minutes.

Place the tubes in a rack. After making certain that no copper reduction has occurred in any of the tubes, add to each tube, properly numbered, 0.5 cc. of spinal fluid. Shake each tube vigorously for 10 seconds.

Replace the tubes in the beaker. Add water halfway to the top. Heat. Keep the tubes in vigorously boiling water for 5 minutes.

Replace the tubes in the rack, inspecting each immediately after removal from the beaker, for precipitate indicating presence of sugar.

Spinal fluids giving a negative reaction for sugar in the above test are unsatisfactory for testing for syphilis. These are fluids that have been acted upon by bacteria either inside or outside of the body. In the former case (bacterial meningitis) in which organisms and ferments of the exudate have acted upon the sugar, the fluids may contain substances that give positive or unsatisfactory reactions in various tests for syphilis. Bacterial contamination subsequent to withdrawal of spinal fluids from cases of syphilis causes a steady loss both of sugar and of the specific reacting substance, if the fluids are kept at room temperature.

On the other hand, if spinal fluids containing sugar when withdrawn from the body are kept at low temperature ( $8^{\circ}$  to  $10^{\circ}$  C.), they continue to give a positive reaction for sugar with the test described above for several weeks, and syphilitic spinal fluids under these conditions show no appreciable loss of specific reacting substance for at least a week.

The clear and cleared spinal fluids which give a positive reaction in the sugar test described above are then tested as follows:

1. Into each of 12 chambers (33 mm. in diameter) on 6 glass slides (in a holder tilted slightly by placing a small metal bar  $\frac{1}{8}$  inch thick under one long slide) deliver 0.05 cc. of 1 per cent glacial acetic acid solution from a 0.2 cc. pipet graduated in thousandths. (It is of the utmost importance that the 1 per cent acetic acid be carefully prepared since this reagent in stronger concentration will precipitate the emulsion.)

2. Into each chamber allow 0.25 cc. of the spinal fluid to be tested (6 spinal fluids in duplicate) to fall from a 1 cc. pipet graduated in hundredths. Hold the pipet directly above the acid and lastly touch the tip of the pipet at some dry portion of the chamber.

3. Rotate the slides in the holder on a flat surface with moderate vigor for 1 minute.

4. Into  $\frac{1}{2}$  of the chambers allow 1 drop (about 0.008 cc.) of diagnostic antigen emulsion to fall from a Wright pipet.

5. Into each of the duplicate spinal fluids 1 drop (about 0.008 cc. of exclusion antigen emulsion) is allowed to fall from a Wright pipet.

6. Rotate the slides in the holder on a flat surface with moderate vigor for 1 minute to distribute the antigen and then for 4 minutes move the holder gently but rapidly (about 3 complete movements a second) back and forth a distance of  $\frac{1}{4}$  to  $\frac{1}{2}$  inch.

7. The results are examined at once through the microscope at a magnification of about 120 times (objective 16 mm. eyepiece 12) with the light cut down as for the study of urinary sediments and recorded in terms of pluses according to the degree of clumping and the size of the clumps. For ease in reading the results, the slide is tilted on a piece of metal  $\frac{1}{8}$  inch thick,  $\frac{1}{2}$  inch wide, and 4 inches long, placed on the stage.

*Readings.*—The results are read at once through the microscope at a magnification of about 120 times (low power 16 mm. objective, eyepiece 12) with the light cut down as for the study of urinary sediments. The results are reported according to the degree of clumping and the size of the clumps (Fig. 309), as ++ (*strongly positive*), + (*positive*), ± (*doubtful*), and — (*negative*).

### MAZZINI MICROSCOPIC FLOCCULATION TEST FOR SYPHILIS \*

**Apparatus.**—1. Serologic *pipets* of 5 cc. capacity to measure the buffered saline solution; 1 cc. capacity, graduated in hundredths to the tip, to measure serum and antigen; and 0.2 cc. capacity, graduated in hundredths, to measure acetic acid.

2. *Bottles* of 30 cc. capacity, round type, 3 cm. in diameter, for preparing one quantity of antigen suspension (3.4 cc.), and 15 cc. bottles, round type, approximately 2.3 cm. in diameter, for preparing half-quantity of antigen suspension (1.7 cc.).

3. *Antigen dropper.* A 5 cc. glass syringe or other convenient tube fitted with a 25 gauge needle.

4. *Serum tube rack.* Any substantially made rack is satisfactory.

5. *Water bath.* A constant temperature 55° C. or 56° C. bath for heating sera.

6. *Serum tubes.* Any convenient size to fit the tube racks.

7. *Centrifuge* of suitable size to the volume of work done.

8. *Rotator.* Rotation by hand is satisfactory provided it is carried out as recommended. Mechanical rotation, however, is superior since it provides a constant and uniform means of agitation.

9. *Microscope* equipped with a low power (16 mm.) objective and a 10X ocular.

10. *Glass slides.* The slide of choice for the serum test is the microscopic slide 2 x 3 inches upon which wax rings have been mounted. For the spinal fluid test it is necessary to employ all glass concavity slides; the concavity should not be less than 2 mm. deep and 16 mm. in diameter.

11. *Slide holder.* Made of any convenient material to accommodate from one to three 2" x 3" slides.

12. *Mounting the wax rings on the glass slides.* The Fisher Rapid Maker, which has a base of 12 metal circles arranged in 3 rows of 4 each, is a convenient instrument for mounting the rings. The wax used is the inexpensive Zubian Sealing Wax employed by "home canners" to seal jars of fruits and vegetables. It is manufactured by Dicks-Pontius Co., Dayton, Ohio, and obtainable at most retail grocery stores. A mixture of the wax and paraffin, in the proportion of 50 gm. wax to 20 gm. paraffin, has been found to give the most satisfactory results. The wax mixture is placed in a

\* *Ven. Dis. Inform.*, 23: 123, 1942.

convenient vessel, such as a pyrex Petri dish, and heated with a microburner from 105° C. to 110° C. and mixed thoroughly. The rings are made by dipping the instrument into the melted wax mixture and then placed on the clean slide for a few seconds. The instrument is then lifted and the process repeated on new slides. If a deeper chamber is desired, a second and third layer may be applied after the wax has been allowed to solidify.

13. *Care of the slides after use.* The wax rings should not be removed from the slides after use. Immediately after reading the tests the slides are placed in a metal slide holder, such as that used in tissue staining, which is kept immersed in distilled water. By this procedure drying of sera on the slides is prevented. After the completion of the tests, the slides are rinsed thoroughly in running tap water and finally rinsed in distilled water. The slides are then transferred to a slide box and allowed to dry at room temperature under cover, or they may be dried by rubbing the chambers of the rings with a soft cloth free from lint. If proper care is given the slides, they may be used indefinitely.

**Preparation of the Antigen Extract.**—1. Twenty grams of dehydrated beef heart powder (Difco), 10 grams of powdered egg yolk \* and 200 cc. of ether for anesthesia are placed in a 500 cc. wide-mouth, glass-stoppered bottle. The mixture is agitated in a mechanical shaker for 5 minutes, or by hand for 15 minutes.

2. The mixture is filtered through a fat-free 24 cm. paper of medium texture into a 500 cc. flask. The ether extraction is repeated 4 additional times using 100 cc. of ether each time. A new filter paper is used for each filtration, and all the ethereal filtrates are collected in the 500 cc. flask. The combined filtrates will be used in step 6.

3. After the last extraction is completed the moist powder is spread on a new piece of filter paper. The powder may be left at room temperature or placed in a 37° C. incubator or allow the ether adhering to the powder to evaporate.

4. The dried powder is then placed in a 500 cc. glass-stoppered bottle, 80 cc. of absolute ethyl alcohol is added and the mixture agitated in a mechanical shaker for 4 hours, or the mixture is left at room temperature for 3 days, being shaken for 5 minutes 3 times each day.

5. The mixture is filtered through a fat-free 24 cm. paper of medium texture into a 100 cc. wide-mouth, glass-stoppered bottle. The powder is now discarded.

6. The combined ethereal filtrates are now placed in a large evaporating dish (8.5 inches in diameter) and the ether evaporated by placing the dish in a water bath at 55° C. until no ether bubbles rise to the surface of the liquid.

7. One hundred cc. of acetone (Merck's Reagent) which has previously been warmed to 55° C. is poured rapidly into the concentrated ether extracts. The mixture is stirred thoroughly with a steel spatula and immediately decanted into two 50 cc. centrifuge tubes, and centrifuged at 2000 r.p.m. for 5 minutes. The acetone is poured off and discarded. Ten cc. of fresh acetone is added to each tube. The acetone-insoluble lipoids are stirred with a glass rod; then placing the palm of the hand over the mouth of the tube it is inverted a few times. The supernatant acetone is poured off and discarded. The acetone-insoluble lipoids are collected with a spatula and added to the alcoholic extract obtained in step 5. The bottle is placed in a 55° C. water bath

\* Powdered egg yolk may be purchased at any reliable wholesale dairy supply company, or from Bessire & Co., Inc., Indianapolis, Indiana.



for 30 minutes, shaking gently at frequent intervals. The extract is then allowed to cool by placing the bottle in the refrigerator for 30 minutes.

8. The extract is filtered through a fat-free 12.5 cm. paper of fine texture. The antigen is then ready for titration. Any precipitate appearing after the extract has stood should be removed by filtration. The antigen is kept tightly stoppered at room temperature and remains unchanged indefinitely.

**Preparation of the 1 Per Cent Cholesterinized Alcohol.**—A sufficient quantity to meet the individual need is prepared. For example: To prepare 50 cc. of the solution, place 500 mg. of cholesterin C.P. (Pfanstiehl Ash Free) and 50 cc. of absolute ethyl alcohol in a 100 cc. glass-stoppered bottle. Heat in the water bath at 55° C. until the cholesterin has completely dissolved; agitate at frequent intervals. Filter the solution through fat-free paper of fine texture.

**Preparation of the Buffered Saline Solution.**—A sufficient quantity of this solution, having a pH of 6.3 to 6.4 and a salt concentration of 10 grams per liter, is prepared. An example based on a 250 cc. volume follows:

Sodium chloride C. P. ....	2.025 gm.
Secondary sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) $12\text{H}_2\text{O}$ .....	0.425 gm.
Primary potassium phosphate ( $\text{KH}_2\text{PO}_4$ ).....	0.050 gm.
Double distilled water .....	250.00 cc.
N/hydrochloric acid .....	0.8 cc.
Formaldehyde (Merck's Reagent) .....	0.25 cc.

The solution is filtered and the pH checked. For practical purposes the colorimetric method of determination is sufficiently accurate. The solution is kept in a glass-stoppered pyrex bottle and will remain unchanged indefinitely. If through carelessness the solution becomes contaminated with particles of dirt, cotton fibers, or other debris, it is necessary to filter the solution because any debris which may be transferred to the suspension will appear in the field of vision during the reading of the test and, unless differentiated from specific flocculate, may lead to false reading.

**Preparation of Serum.**—The patient's serum is separated from the clot by centrifugalization and heated for 30 minutes in the water bath at 55° C. to 56° C. For emergency pretransfusion test the serum may be heated at 60° C. for 10 minutes. Inspection of sera for visible precipitate after heating should be done as a matter of routine. Occasionally heated serum throws off a precipitate which, if not removed by recentrifugalization, may interfere with serologic reactions in general. There is not, however, the possibility that the precipitate, if overlooked and left in the serum, will be mistaken for the true flocculate of a positive reaction when read microscopically, which does occur when specimens containing this pseudoprecipitate are read macroscopically. Another potential source of error to the inexperienced is the occasional presence of oil globules which have been transferred from lubricated syringes at the time the blood was drawn. The globules grouping themselves in clusters of varying sizes simulate weakly and even strongly positive reactions. Specimens containing an excessive amount of oil should be reported as unsatisfactory and a new sample requested. If it is necessary to retest a weakly positive reacting serum which has been heated and thereafter kept at room temperature or in the refrigerator for several hours, it is advisable to draw off a new portion of the serum from the clot. In strongly positive sera reheating for 15 minutes does not cause sufficient destruction of reagin

to make an appreciable change. Hemolyzed sera can be tested with accuracy provided they are not excessively viscous after the heating period.

**Determination of the Optimum Lipoid-Cholesterin Ratio.**—1. Set up 5 clean and dry serologic test tubes in a rack. Label them 1 to 5.

2. Place 0.1 cc. of the antigen extract directly into the bottom of each tube.

3. Add 0.9 cc., 1.4 cc., 1.9 cc., 2.4 cc., and 2.9 cc. of 1 per cent cholesterinized alcohol to each of the 5 tubes respectively. Mix thoroughly the contents of each tube. Tube 1 contains a 1:10 ratio; tube 2, 1:15; tube 3, 1:20; tube 4, 1:25; and tube 5, 1:30.

4. Take five 30 cc. bottles and label them 1:10, 1:15, 1:20, 1:25, and 1:30, respectively.

5. Pipet 3 cc. of buffered saline solution into each of the 5 bottles.

6. With a 1 cc. pipet, graduated to the tip, measure 0.4 cc. (reading from the bottom of the pipet) of the 1:10 cholesterinized antigen; hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the buffered saline from the pipet held in the right hand. Draw the suspension into the pipet and blow in and out two or three times.

7. Proceed in the same manner in preparing the 1:15, 1:20, 1:25, and 1:30 suspensions. The bottles are corked and allowed to stand at room temperature for 3 hours without further manipulation.

**Trial of the Suspensions.**—1. At the end of 3 hours, suspension 1 is shaken gently from bottom to cork and back 10 times, then it is poured into a 5 cc. syringe fitted with a 25 gauge needle.

2. Select 30 sera from cases known to be free from syphilitic infection.

3. Place 0.05 cc. of serum from each of the 30 negative specimens in the corresponding one of the 30 chambers of 3 glass slides which have been placed on a slide holder.

4. Discharge 1 drop of the 1:10 suspension into each of the 30 sera.

5. Rotate the slides with a circular, slightly "jerky" motion for 4 minutes at 120 rotations per minute. It is important that the number of rotations be that indicated and that the proper motion be given to the slides to insure that the antigen particles become well dispersed throughout the area of the rings. It is not necessary that the motion be of such nature as to cause the sera to "jump" the rings.

6. Examine the results under the low power (16 mm.) objective of the microscope with subdued light. Record the results. Every one of the sera should show numerous, very small, round or slightly elongated particles of lipoid-cholesterin complex. These particles uniformly dispersed throughout the field should not show the slightest clumping.

7. Place 3 other slides on the holder and using the same sera as previously used proceed to try out the 1:15 suspension. Follow this with the 1:20, the 1:25, and finally with the 1:30 suspension. Record the results. Usually within the range of 1:10 to 1:30 will be found one or more ratios in which the cholesterin is in excess of the lipoids, allowing the spontaneous clumping of the particles. Obviously these ratios cannot be employed in the test proper since false positive reactions will be obtained with such suspensions.

**Determination of the Antigenic Quality of the Suspension.**—Having already determined the lipoid-cholesterin ratios which will not cause false positive reactions

with negative sera, the next step in the standardization of the antigen is the evaluation of the antigenic properties of these suspensions. For this purpose select at least 10 partially positive sera, preferably those from long-treated cases. The object is to employ sera containing as few reacting units as possible.

1. Place 0.05 cc. of serum from each of the 10 partially positive specimens into the corresponding chambers of a glass slide.

2. Discharge 1 drop of the 1:10 suspension into each serum. Rotate the slide for 4 minutes at 120 rotations per minute. Examine through the microscope. Record the results.

3. Proceed to try out the rest of the suspensions which were found to give clear-cut negative reactions with known negative sera by following the same procedure as for the 1:10 suspension. When these trials are conducted with sera containing relatively few reacting units, it will be observed that the lower the lipid-cholesterin ratio the weaker the reaction, the flocculate increasing in size as the ratio increases. After recording all the results and having made a study of them, a final lipid-cholesterin ratio is selected which is designated as the titer of the antigen. For *maximum sensitivity*, the suspension containing the highest lipid-cholesterin ratio which *does not cause the least clumping in the presence of negative sera* is selected as the titer. If a less sensitive antigen suspension is desired a lower ratio is chosen. Naturally, a greater degree of safety is obtained by using a lower ratio but the sensitivity usually will be decreased.

When the titer of the antigen has been determined, a sufficient amount of cholesterinized antigen is prepared to meet the individual need for approximately 1 month. For example: If the ratio selected is that of 1:20, then by taking 0.5 cc. of the antigen extract and adding 9.5 cc. of 1 per cent cholesterinized alcohol, a supply for about 1 month is obtained, since 0.4 cc. of the cholesterinized antigen, regardless of the ratio, is the fixed amount to use in 3 cc. of buffered saline solution. This volume (3.4 cc.) will be sufficient for about 300 tests. The cholesterinized antigen keeps unchanged for several months provided it is tightly stoppered. If evaporation of alcohol from the antigen is allowed to take place, it results in the precipitation of cholesterin, making the antigen unfit for use.

It would seem that the antigenic determination could be eliminated and the titer be based solely on the lipid-cholesterin ratio. By this procedure less time and labor would be involved in the standardization of the antigen. Experience has shown, however, that this determination is very desirable if not essential. Sometimes it can be demonstrated that two different ratios give approximately the same degree of flocculation with weakly positive sera, and while there is little choice between the two, the logical dilution to employ is the one having the lower ratio, because the sensitivity is the same while the margin of safety (specificity) is increased. Obviously if the antigenic determination had not been carried out the dilution of choice as determined by the lipid cholesterin ratio titration would be the higher of the two. *It is possible to eliminate all titrations and to set an arbitrary mean which past experience has shown in the average lipid-cholesterin ratio.* For instance, the most frequently encountered ratios which give clear-cut negative reactions with negative sera are the 1:10, 1:15, 1:20 and 1:25. Therefore, an arbitrary selection of the dilution containing the 1:15 or 1:20 ratios could be made provided strict adherence to details of technic is observed. Nevertheless, if the highest sensitivity consistent with safety is to be

obtained it follows that both titrations are necessary. In general, the accuracy with which serologic reagents are standardized largely determines the quality and correctness of the work performed in any laboratory irrespective of the merits of the technic involved.

**Qualitative Test with Serum.**—1. Pipet 3 cc. of buffered saline solution into a 30 cc. bottle.

2. With a 1 cc. pipet, graduated to the tip, measure 0.4 cc. (reading from the bottom of the pipet) of the cholesterinized antigen; hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the saline solution from the pipet held in the right hand. Draw the suspension into the pipet and blow in and out 2 or 3 times. The bottle is corked and allowed to stand at room temperature for 3 hours, at which time the suspension reaches its optimum sensitivity, or the bottle may be placed in the refrigerator at 6° C. to 8° C. for 15 minutes to accelerate the ripening of the antigen suspension and then it can be used immediately. However, at this stage of ripening it will occasionally fail to react with weakly positive serum. The suspension continues to be usable for 24 hours, after which it decreases in sensitivity; dependable results require, therefore, that it should be used within this period of time. If the number of tests to be performed is small  $\frac{1}{2}$  quantity of the reagents can be prepared.

3. At the end of 3 hours at room temperature, or 15 minutes in the refrigerator, the suspension is shaken gently from the bottom to cork and back 10 times and transferred to a 5 cc. glass syringe fitted with a 25 gauge needle and is then ready for instant use. It has been ascertained that some antigens do not disperse completely when added to the buffered saline solution, hence it is advisable that the suspension be shaken gently, yet thoroughly, after the "ripening" period in order to obtain a "smooth" suspension.

4. Place 1, 2, or 3 glass slides on a slide holder, depending on the number of specimens to be tested. Pipet 0.05 cc. of each patient's serum, which has previously been heated for 30 minutes at 55° C. to 56° C., into the corresponding chamber of the glass slide. Discharge 1 drop of the ripened antigen suspension into each of the sera in the chambers. Known negative and positive sera as controls on the antigen should be included.

5. Rotate the slide holder for 4 minutes at 120 rotations per minute.

6. First examine every one of the rings macroscopically to make certain that no serum has "jumped" the ring and contaminated another. Then examine the results microscopically under the low power objective (16 mm.) with subdued light. Inspection of the periphery of the rings for clumps should be made a routine practice, for occasionally the flocculate is very compact and has a tendency to locate in the outer portion of the ring. Record the results as follows: No clumping, negative; very small clumps, 1+; small clumps, 2+; medium size clumps, 3+; large clumps, 4+.

An alternative method of reading may be used as follows: No clumping, negative; very small to small clumps, doubtful; medium to large clumps, positive.

It should be obvious that every specimen found to react positively, whether it be strongly positive, moderately positive, or weakly positive, must be retested before being reported if mechanical errors are to be excluded.

In reading weakly positive reactions care should be taken to differentiate red blood cells, oil globules or debris (which may be contained in the serum, or in the slide, or

in the antigen suspension) from the true flocculate of a positive reaction. The reading of any serologic test requires judgment and experience since no accurate standard can be prepared. Although no experience is needed to read strongly positive reactions, time and observation alone will lead to correct interpretation of weakly positive reactions.

Zone reactions are usually due to an off balance in the antigen-reagin ratio, although surface area and perhaps other factors play a role in causing these reactions. There are 2 general types of zone reactions as they apply to this: In one the cause is an insufficient amount of antigen, and in the other it is due to an excess of reagent. In the majority of cases of type I zones, the phenomenon is readily recognized by the appearance of irregular aggregates. These clumps, varying in size from small to large floccules, are scattered in fields in which smaller clumps predominate. The correction of this deficiency is effected by adding a second drop of antigen suspension to the serum and rerotating the slide for 4 minutes. If the addition of the second drop of antigen fails to increase materially the degree of flocculation, then the reaction is probably a type II zone. The second type of zone reaction is one seldom encountered, but it is more difficult to detect because of the almost complete inhibition of flocculation, and usually there is also absence of irregular aggregation. Zone reactions of this type are corrected by preparing serial dilutions of the serum, such as 1:2, 1:4, 1:8, 1:16, etc., and proceeding as for routine testing. *Since zone reactions are frequently misinterpreted and at times not even recognized, it is recommended that the addition of a second drop of antigen be made a routine practice whenever weakly positive reactions are obtained.*

**Quantitative Test with Serum.**—The procedure for the quantitative test is exactly the same as for the routine test with the exception that each serum is tested in serial dilutions.

Set up 6 empty tubes (more if necessary) in a row in a rack. Place 0.5 cc. of the heated serum to tube 1, add 0.5 cc. of physiologic saline solution, mix thoroughly, transfer 0.5 cc. to tube 2, add 0.5 cc. saline solution and continue the process until all the dilutions have been made.

The results may be reported in terms of the actual quantity of serum tested, for example: 0.025 cc., 4+; 0.0125 cc., 4+; 0.0062 cc., 4+; 0.0031 cc., 2+; 0.0015 cc., 1+; and 0.0007 cc., negative; or they may be reported in terms of positiveness according to the highest dilution giving a positive reaction, such as positive up to a 1:16 dilution.

**Qualitative Test with Spinal Fluid.**—Spinal fluid should be tested as soon as possible after it is drawn. A grossly contaminated fluid is not dependable with this or any other test. It should not contain blood in appreciable quantity. The heating of spinal fluid is neither necessary nor desirable.

**Rapid Test.**—1. Centrifuge the fresh fluid at 2,000 r.p.m. for 5 minutes, pour off the clear supernatant fluid into a clean tube.

2. With a 0.2 cc. pipet graduated in hundredths place exactly 0.01 cc. of 6 per cent acetic acid in one side of as many chambers of a glass slide as there are fluids to be tested. Accurate preparation of the acid solution and the amount delivered to each chamber is obviously necessary, for stronger concentrations or larger amounts will flocculate the suspension even in the absence of reagin.

3. With a 1 cc. pipet deliver 0.1 cc. of spinal fluid in the opposite side of the

chamber from where the acid is located. Mix the acid and fluid evenly over the surface of the chamber with a wood applicator.

4. Rotate the slide holder with a circular motion for 1 minute. Thorough mixing of the acid and the fluid is essential.

5. Add 1 drop of the same antigen suspension, as is used for testing serum, to each chamber containing spinal fluid.

6. Rotate the slide holder for 10 minutes at 120 rotations per minute. The results are examined and recorded in the same manner as for the test with serum.

**Concentration Test.**—If a moderately positive (3+) or a strongly positive (4+) result is obtained with the acid test, no further testing is necessary; the weakly positive and negative reacting fluids are subjected to the concentration test. The concentrating apparatus consists of a hot air blower—the ordinary hair dryer is satisfactory and inexpensive—which has been clamped on a steel rod support.

1. Place about 50 cc. of cold water in a Petri dish.

2. Pipet 1.5 cc. of spinal fluid into the bottom of a 50 cc. beaker (4 cm. inside diameter).

3. Set the beaker in the center of the Petri dish containing the water.

4. Place the blower at a distance of 2 cm. above the beaker and turn on the level of the dryer to the "hot air" position.

5. Evaporate the fluid to a volume of 0.2 cc. to 0.3 cc. This is accomplished in 6 to 8 minutes.

6. Proceed to test the concentrated fluid in exactly the same manner as that used in the acid test.

**Quantitative Test with Spinal Fluid.**—Serial dilutions as those prepared for the quantitative test with serum are made and the acid test only is carried out.

### PRECIPITIN TESTS FOR THE IDENTIFICATION AND DIFFERENTIATION OF BLOOD STAINS AND OTHER SUBSTANCES

These tests are usually required for medicolegal purposes and especially for the identification of human blood.

**Preparation of Immune Serum.**—1. Immunize several rabbits by *intravenous* injections of human serum at 3-day intervals in the following amounts: 1, 2, 3, 4, 8, and 10 cc. or inject at 5-day intervals in the following amounts: 2 doses of 8 cc. each, 2 of 5 cc., and 2 of 3 cc. To prevent anaphylactic shock the rabbits may be desensitized by injecting 0.2 cc. of the serum  $\frac{1}{2}$  hour before injection of each of the later doses. Ten days after the last dose remove a small amount of blood from an ear vein and test the serum for precipitin by placing 0.2 cc. in a small test tube and carefully overlaying with 1 cc. of a 1 : 1000 dilution of human serum. If a ring of precipitation occurs in a few minutes the *fasting* animal may be bled from the heart aseptically in sterile centrifuge tubes. After clotting has occurred, *gently* separate the clot and centrifuge to remove all corpuscles. Transfer the serum to sterile containers and to each 19 cc. add 1 cc. of 1 : 500 metaphen solution for preservation. For use the serum must be crystal clear for accurate results and this may require centrifugalization or filtration.

If the preliminary test is negative give the animal 3 additional doses of serum

*intraperitoneally* in the following amounts: 10, 15 and 20 cc. Test again 10 days after the last dose.

For medicolegal work it is advisable to prepare similar precipitins for the sera of the commoner domestic animals as the hog, sheep, beef, chicken, dog, cat, etc.

**Preparation of Unknown Material.**—Prepare a solution of the stain or other material in normal saline solution. Apply a test for blood like the benzidine, hemin crystal or other procedure. The extract should be approximately 1:1000 and fulfill the following requirements: (a) crystal clear and almost colorless; (b) contain only a very small amount of protein when tested by the heat and acetic acid or nitric acid ring tests; (c) foam freely on shaking; (d) be neither strongly acid nor strongly alkaline to litmus paper.

If the solution of stain was from a piece of cloth, leather, or other material prepare a similar extract of an unstained portion as a control. Of course, it may not contain protein.

**Method.**—1. Arrange 7 small test tubes or preferably pointed capillary tubes of about 3 mm. inside diameter and charge them as follows:

No. 1.—0.2 cc. immune serum overlayed with 1 cc. of unknown extract.

No. 2.—0.2 cc. normal rabbit serum overlayed with 1 cc. of unknown extract (negative control).

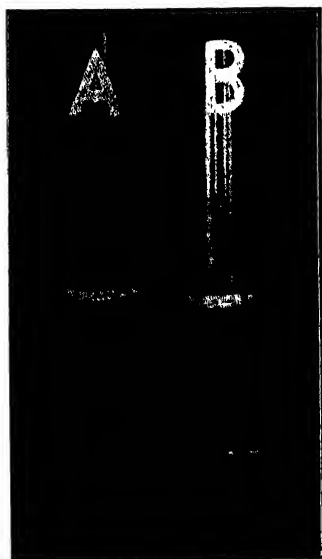


FIG. 310.—PRECIPITIN REACTIONS

A, positive; B, negative.

No. 3.—0.2 cc. of immune serum overlayed with 1 cc. of a 1 : 1000 solution of sheep or other serum different from that suspected in the stain of unknown material (negative control).

No. 4.—0.2 cc. immune serum overlayed with 1 cc. of 1 : 1000 dilution of human serum (positive control).

No. 5.—0.2 cc. immune serum overlayed with 1 cc. of control extract if one has been prepared of unstained cloth, leather, etc. (control).

No. 6.—0.2 cc. immune serum plus 1 cc. of normal saline solution (control).

No. 7.—0.2 cc. saline solution plus 1 cc. of extract (control).

2. Allow to stand at room temperature for 5 to 15 minutes and examine.

3. White rings of precipitation at the lines of contact in tubes 1 and 4 with no reactions in the other

tubes indicates that the extract of unknown material contained human protein and if the chemical reactions were positive for blood would indicate that the extract was of human blood (Fig. 310). The test, however, does not differentiate the blood of one human being from another. Doubt can arise only between the proteins of closely related species, as for example, man and the higher apes, sheep and goat, horse and mule, etc.

4. If the tests are negative for human blood duplicate tests may be conducted

with anti-chicken, anti-beef, anti-dog, anti-cat and other immune sera. In medicolegal cases it is always advisable to conduct these even though positive reactions are observed with anti-human serum.

5. Similar tests can be conducted for the detection of meat adulteration, identification of bones, milk, semen, etc., the technic being given in Kolmer's *Infection, Immunity and Biologic Therapy*, W. B. Saunders Company.

### PRECIPITIN TESTS IN MENINGOCOCCUS MENINGITIS

This test described by Kreidler and Murphy is not only of diagnostic aid in differentiating meningococcus from other types of suppurative meningitis, but it is also of service in the selection of antimeningococcus serum for treatment, preference being given to the serum showing the maximum of precipitation.

1. Centrifuge the spinal fluid at high speed for 20 minutes.
2. Remove the clear supernatant fluid to another test tube. The sediment may be used for smears and cultures.
3. Place 0.3 to 0.5 cc. of each available antiserum in small test tubes.
4. Carefully overlay each with an equal volume of the clear spinal fluid.
5. Allow to stand at room temperature for 20 minutes. Examine for rings of precipitation at the lines of contact which constitute positive reactions graded from + to + + + +.

**For Meningococcus Polysaccharide in Spinal Fluid.**—The presence of meningococcus polysaccharide in the cerebrospinal fluid is indicative of a severe infection and the need for adequate treatment with sulfadiazine and anti-meningococcal serum. A test for the polysaccharide may be conducted as follows:

1. Place 0.2 cc. of perfectly clear polyvalent or type-specific anti-meningococcal serum in a small test tube.
2. With a fine capillary pipet, carefully overlay with 1 cc. of perfectly clear centrifuged spinal fluid.
3. A positive reaction is indicated by the formation of a white ring of precipitate at the line of contact. If occurring within 10 minutes at room temperature, it is usually indicative of a severe infection.

### PRECIPITIN TESTS FOR PNEUMOCOCCUS POLYSACCHARIDE

Recovery from pneumococcal pneumonia, meningitis and other infections is largely dependent upon the presence in the body of adequate amounts of type-specific antibody. In the presence of inadequate amounts of the latter the soluble specific capsular polysaccharide of the infecting pneumococcus is not neutralized and may occur in the serum, cerebrospinal fluid or urine. Under the conditions, tests for this type-specific polysaccharide are of value in relation to gauging the severity of infection and treatment with sulfadiazine and specific serum, with special reference to pneumonia and meningitis. The precipitin test for the detection of the type-specific polysaccharide in the blood serum, urine or cerebrospinal fluid (in meningitis) is usually employed. It may be conducted as follows:

1. Place 0.2 cc. of clear rabbit type-specific antipneumococcus serum in a small test tube.



2. With a fine capillary pipet, carefully overlay with 1 cc. of perfectly clear spinal fluid, urine or blood serum.

3. A positive reaction consists in the formation of a white ring of precipitate at the line of contact. If occurring within 10 minutes at room temperature, it is indicative of a severe infection with the presence of free type-specific polysaccharide in the fluid being tested. The significance of a positive reaction, therefore, is just the opposite to that of a positive skin reaction in relation to prognosis and treatment.

### **PRECIPITIN TESTS FOR *HEMOPHILUS INFLUENZAE* POLYSACCHARIDE**

*Hemophilus influenzae* belonging to type B is also known to produce a type-specific soluble polysaccharide. Recovery from severe infections with the bacillus, as in influenzal meningitis and pneumonia, is due largely to specific antibody supplemented by sulfonamide therapy. The presence of the polysaccharide in the cerebrospinal fluid, blood or urine is indicative of severe infection with inadequate amounts of antibody in the body and may be detected by precipitin tests. These tests may be conducted with blood serum, urine or cerebrospinal fluid as follows:

1. Place 0.2 cc. of clear type B anti-influenzal serum in a small test tube.
2. With a fine capillary tube, carefully overlay with 1 cc. of perfectly clear fluid to be tested.
3. The appearance of a white ring of precipitate at the line of contact within 10 minutes at room temperature, is believed to indicate a severe infection with insufficient antibody in the body for the neutralization of the excess polysaccharide shown in the cerebrospinal fluid, serum or urine being tested.

### **PRECIPITIN TEST FOR PLAGUE**

1. This test is sometimes of value in making a rapid presumptive diagnosis on decayed tissues and especially of rats.

2. One part of finely divided tissue is mixed with 5 to 10 parts of distilled water, boiled for 5 minutes and filtered repeatedly through paper or asbestos wool with suction until perfectly clear. Set up the tests in small test tubes:

No. 1.—Place 0.5 cc. of high titer immune serum and carefully overlay with 0.5 cc. of the filtrate.

No. 2.—Set up a duplicate using normal rabbit or horse serum (control).

No. 3.—Place 0.5 cc. of immune serum and overlay with 0.5 cc. of normal saline solution (control).

No. 4.—Place 0.5 cc. of extract and overlay 0.5 cc. of normal saline solution (control).

3. A positive reaction shows a white ring of precipitate at the line of contact in the first tube in 5 minutes at 37° C. reaching a maximum after 2 hours. A negative reaction is not conclusive.

**PRECIPITIN TEST FOR ANTHRAX**

A similar test may be conducted with an extract of tissue and a potent anti-anthrax serum.

**PRECIPITIN TEST FOR ECHINOCOCCUS DISEASE**

This test is frequently of diagnostic aid. The antigen is the clear fluid from a hydatid cyst. The serum is that of the patient which should be crystal clear, undiluted and used unheated.

1. Set up the tests in small test tubes as follows:

No. 1.—0.5 cc. of patient's serum carefully overlayed with 0.5 cc. of antigen.

No. 2.—0.5 cc. of normal serum carefully overlayed with 0.5 cc. of antigen (negative control).

No. 3.—0.5 cc. of serum from a rabbit immunized by intravenous injections of cyst fluid or from a known case of the disease carefully overlayed with 0.5 cc. of antigen (positive control).

No. 4.—0.5 cc. of patient's serum with 0.5 cc. of saline solution (control).

No. 5.—0.5 cc. of antigen with 0.5 cc. of saline solution (control).

2. Allow to stand at room temperature for  $\frac{1}{2}$  hour. A white ring of precipitation in tubes 1 and 3 indicates a positive reaction.

**PRECIPITIN TEST FOR TRICHINOSIS**

This test is particularly valuable in checking diagnoses made by skin tests in patients suspected of harboring the encysted larvae of *Trichinella spiralis*. The antigen may be the same as that employed for the skin test prepared according to the method of McNaught, Beard and Myers (*Am. Jour. Clin. Path.* 11: 195, 1941). The test is performed by carefully overlaying 0.2 cc. of a 1:100 dilution of antigen over 0.2 cc. of clear unheated serum in a narrow test tube. The control consists of layering saline solution over the serum. The tubes are placed in a water bath at 37° C. for an hour. A distinct opaque whitish 1 mm. ring at the junction of the antigen and serum indicates a positive reaction.

## METHODS FOR CONDUCTING IMMUNOLOGIC AND ALLERGIC SKIN TESTS

The only immunologic skin tests commonly employed are those of Schick and Dick for natural or acquired immunity to diphtheria and scarlet fever respectively, including the Schultz-Charlton blanching test sometimes of value in the diagnosis of scarlet fever. To these may be added the skin tests for the presence or absence of antibody in pneumococcal pneumonia and meningitis; also in *Hemophilus influenzae* infections. Possibly the intradermal injection of antitularia serum (Foshay), employed as an aid in the diagnosis of tularia, should be included in this category although its mechanism is unknown at the present time.

Skin tests for natural or acquired allergy, however, are widely employed in the etiologic diagnosis of numerous allergic diseases with special reference to seasonal hay fever and asthma, vasomotor or allergic rhinitis, perennial or bronchial asthma, allergy to foods (gastro-intestinal allergy), the allergic dermatoses, migraine, contact dermatitis, serum and drug allergy; also for allergy in various bacterial diseases (tuberculosis, undulant fever, tularia, glanders, etc.) for diagnostic purposes as well as in some of the mycotic diseases and those due to animal parasites.

### TECHNIC OF CUTANEOUS TESTS

*Cutaneous or scratch tests* have the advantages of simplicity and inexpensiveness since 30 or more may be conducted at one time. They are also highly specific with nonspecific reactions of rare occurrence. They are likewise safe since the absorption of amounts of allergens sufficient for the production of constitutional reactions does not occur. Their one important disadvantage is a lack of sensitivity. However, they are usually sufficiently sensitive for tests with the inhalant allergens in hay fever, asthma and allergic rhinitis; furthermore, they are frequently preferred in testing suspected cases of natural allergy to horse or other animal sera in which hypersensitivity may be so exquisite as to render intracutaneous tests dangerous.

1. These tests are done preferably upon the skin of the forearm or inner aspect of the arm in adults, and upon the skin of the back in infants or young children. The skin is cleansed with alcohol and dried. A small superficial abrasion about  $\frac{1}{8}$  inch long is made through the epidermis by a small needle, a sharp pointed scalpel or a lancet (Fig. 311). Care should be taken not to draw blood. When more than one test is being done, the entire series of scratches should be made at one time in rows of 4 to 8. The scratches should be spaced at least  $\frac{1}{2}$  inch apart to insure clear definition of positive reactions. One of the scratches should be kept for a control—this usually is placed in the cubital fossa between the linear rows.

2. If the allergen is a liquid or a paste, it may be applied directly to the scratch. Very little rubbing is necessary following the application, since the scratch will usually absorb the material applied to it. If the allergenic extract is in powder form, a drop of a diluent which may be either tenth-normal sodium hydroxide or normal salt solution should be placed upon each scratch. The powdered extract is then added to the diluent from the blunt end of a toothpick employing just enough of the powder to cover the end of the toothpick. This should then be mixed with the diluent by means of the toothpick and allowed to remain on the scratch (Fig. 312). The control site should receive only the drop of diluent.

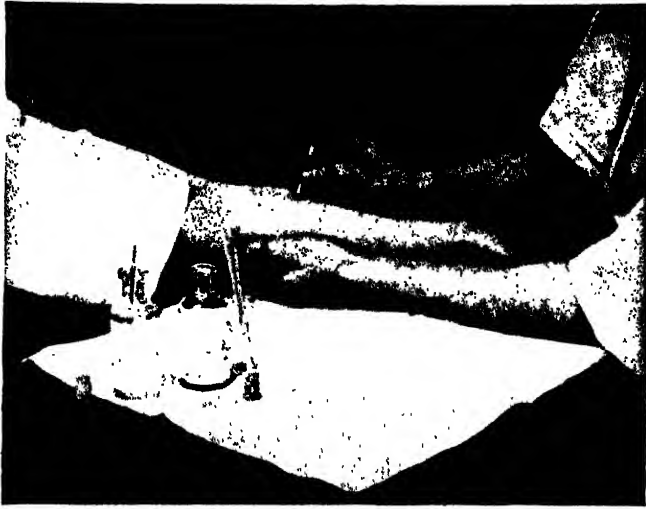


FIG. 311.—METHOD OF MAKING SCARIFICATIONS FOR CUTANEOUS ALLERGIC TESTS WITH DALAND LANCET

(From Kolmer, *Infection, Immunity and Biological Therapy*, 3d ed., W. B. Saunders Co., Philadelphia.)



FIG. 312.—METHOD OF APPLYING ALLERGENS TO CUTANEOUS ABRASIONS IN ALLERGIC SKIN TESTS

(From Kolmer, *Infection, Immunity and Biological Therapy*, 3d ed., W. B. Saunders Co., Philadelphia.)

3. Reactions usually reach their maximum in from 15 to 30 minutes with an average of 20. The material should therefore be left on the scratch until the reaction develops and then wiped off, so that the reaction may be read. Reactions may develop rather quickly in highly sensitive individuals and it is advisable to wipe off the material as soon as they are observed. This will prevent the remote possibility of a constitutional reaction.

Reactions may be recorded as follows (Plate XVI):

Negative (—)	No appreciable increase in size of wheal or areola as compared to control.
Doubtful (±)	Wheal less than 0.5 cm., slight to moderate areola or area of redness.
Slightly positive (+)	Wheal 0.5 cm.; moderate areola.
Moderately positive (+ + +)	Wheal 0.5 to 1 cm. and without pseudopodia; moderate to marked areola.
Markedly positive (+ + +)	Wheal 1 cm. or more with pseudopodia and moderate to marked areola.

### TECHNIC OF INTRACUTANEOUS TESTS

*Intracutaneous tests* have the great advantage of sensitivity and are, therefore, preferred in tests for the detection of hypersensitiveness to foods, bacteria, serum, fungi and animal parasites. The reactions are also quicker and generally easier to interpret. But they are more likely to yield local nonspecific and constitutional reactions, and are more time-consuming and expensive since not more than 6 to 18 tests can be conducted at one time.

1. The skin of the outer aspect of the arm has been found more satisfactory and convenient for this type of testing than any other part of the body, although the skin of the forearm or back likewise may be employed when necessary. It is important that the arm or forearm be freed of any constricting bands, such as is often formed by rolling up a tightly fitting sleeve. The obstruction of the venous circulation produced by this "cuff" tends to inhibit or lessen the intensity of the skin reaction. The skin is cleansed with alcohol and dried. A very small amount (0.01 to 0.02 cc.) of the sterile liquid allergen (dilute extracts usually employed for routine testing) is injected by a tuberculin syringe into the outer layer of the skin (Fig. 313). Care should be taken that the needle is introduced into and not through the epidermis. This may be obviated by introducing the bevel of the needle far enough to pick the skin up with the point and then injecting the liquid extract. Following the injection of the material a small whitish pinpoint elevation or wheal should be visible. As in the scratch technic, the tests should be arranged in rows of 4 to 6, with at least  $\frac{1}{2}$  inch between tests. Six to 12 tests usually are performed at a sitting and although a large number may be done at one time without danger, it is not advisable, particularly with pollens or inhalants, because of the greater danger of constitutional reactions. The number of tests for young children at one time varies between 4 and 8.

2. Reactions are usually complete in from 5 to 15 minutes, with an average of 10 minutes, and should be read at the end of that period. An injection of the diluent constitutes the control site although in most instances a negative reaction may be

## PLATE XVI



### SKIN REACTIONS AFTER SCRATCH TESTS WITH ALLERGENIC EXTRACTS

*a*, — (negative or control); *b*,  $\pm$  (doubtful); *c*, + (slight); *d*, ++ (moderate); *e*, +++ (marked).

(From Kolmer and Tuft, *Clinical Immunology, Biotherapy and Chemotherapy*, W. B. Saunders Co., Philadelphia.)



used as the control. It is advisable, whenever possible, to recheck all positive reactions, employing the next higher concentration of the extract for those allergens giving only doubtful or slight reactions upon skin test.

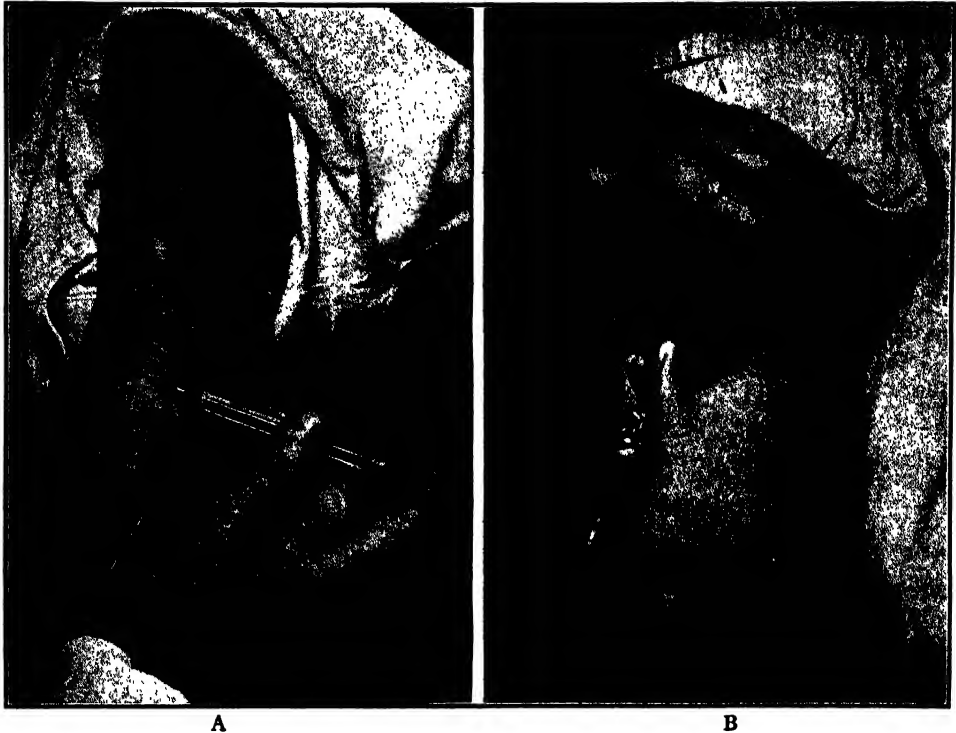


FIG. 313.—TECHNIC OF INTRACUTANEOUS INJECTIONS

A, by tuberculin syringe in upper arm (from Kolmer and Tuft, *Clinical Immunology, Biotherapy and Chemotherapy*, W. B. Saunders Co., Philadelphia); B, by Record syringe in upper arm (note wheal indicative of correct injection). (From Kolmer, *Infection, Immunity and Biologic Therapy*, W. B. Saunders Co., Philadelphia.)

3. The readings are made in the same manner as in the scratch or cutaneous tests. In some patients there may be no immediate reaction to the skin test, but within several hours (usually within 24 hours) there appears about the site of the test a zone of redness or erythema of varying size and with a tendency to persist for a longer period than the immediate skin reaction. This is known as a delayed reaction, and although it is recorded in the same way as immediate reactions, its exact significance is not known. Positive delayed skin reactions to food allergens are of occasional clinical importance.

### TECHNIC OF CONJUNCTIVAL TESTS

*Ophthalmic or conjunctival tests* are less sensitive than intracutaneous tests; hence they never give positive reactions when the latter are negative. On the other hand, however, positive ophthalmic and intracutaneous reactions indicate a higher degree of



hypersensitiveness than a positive intracutaneous reaction alone. Consequently they are particularly useful tests for allergic sensitization to sera preliminary to their administration in the prophylaxis and treatment of disease since a positive ophthalmic reaction indicates the need for great care. They are also sometimes used in testing for pollen allergy but only under special conditions. Needless to state, they cannot be employed in the presence of conjunctivitis nor in the case of crying children or adults.

One or 2 drops of the solution to be tested, or a minute amount of dried extract (usually pollen), is instilled into the conjunctival sac of one eye, the opposite eye being used as a control. Reactions, if positive, occur within 5 to 10 minutes and are characterized by a definite redness due to injection of the conjunctival vessels and by considerable itching and lacrimation. Ordinarily, the reaction disappears within a few hours. When the reaction is marked, swelling of the conjunctiva or of the lower lid may occur and persist for 24 or 48 hours. Uncomfortable reactions are controlled readily by instillation of a drop or two of adrenalin (1:1000) into the eye. Reactions are read in the same manner as skin tests, although the degree of the reaction is more difficult to determine.

### TECHNIC OF NASAL TESTS

*Nasal tests* have a very limited clinical application. They are of occasional value in allergic rhinitis in determining the clinical significance of certain inhalant substances giving doubtful skin reactions. They are also useful at times in detecting sensitivity in hay fever patients with positive clinical histories but negative skin tests, or, like the conjunctival test, in differentiating the clinical significance of tests to pollens with overlapping pollination periods.

This test is performed either by spraying the liquid allergen into the nose or by holding the powdered allergen (e.g., pollen) close to the nostrils upon the blunt end of a toothpick and having the patient inhale it, or by direct application of the allergen to the nasal mucosa. The reaction will manifest itself in sensitive patients by subjective symptoms such as sneezing and watery discharge, or cough. Proper controls should be used to exclude a possible nonspecific reaction.

### TECHNIC OF THE PATCH OR CONTACT TEST

This test is employed in the determination of the specific exciting agent responsible for certain inflammatory conditions of the skin grouped under the term of contact dermatitis and best illustrated by poison ivy. It is the only test of any value in the diagnosis of these conditions and is at times an invaluable aid.

Since the eruption of contact dermatitis is the result of an acquired sensitization, the direct application of the suspected excitant to the skin of such a patient should, after sufficient contact, reproduce a lesion similar to that for which the patient is being treated.

1. Prepare a patch consisting of adhesive plaster 1 to 2 inches square. Cover all of the inner or adhesive surface of the patch except for a rim of about  $\frac{1}{4}$  inch with a square of cellophane, rubber tissue or oiled silk.

2. Prepare a small square ( $\frac{1}{2}$  inch) of linen or blotting paper to be used for

absorbing the liquid or ointment preparations. Solid substances may be applied directly to the skin.

3. The skin of the outer surface of the arm or inner surface of the forearm should be employed when only a few tests are to be done at one time. When 10 to 20 are applied, the skin of the back is more convenient.

4. To perform the test, soak the small square of linen or blotting paper in the liquid to be tested and apply it directly to the skin. If the suspected agent is an ointment, this may be put on the square intact; if it is a powder, it may be moistened with saline or distilled water and then put on. Solid materials may be put on directly without any square. Cover the square with the patch, prepared as described. If the edges of the patch are not firm enough, seal them by adhesive strips or collodion.

5. Allow the patch to remain in place 24 hours, unless itching is marked. With less potent excitants it may be allowed to remain 48 hours or longer.

6. A positive reaction is indicated by the presence at the site of the patch of a reddened area in which small blisters or vesicles are seen. A negative reaction shows no changes at the site. The area should be inspected daily for 2 weeks for signs of a reaction, before being considered negative.

7. A positive reaction is definite indication of sensitization. Its clinical importance must, however, be established. A negative reaction does not necessarily exclude the presence of sensitization.

### TECHNIC OF THE INDIRECT TEST

The *passive transfer or indirect test* is based upon the demonstration of allergic antibody or reagin in the blood of the patient. It is of occasional value when cutaneous or intracutaneous tests are impossible or inadvisable, as in severe universal eczemas and especially in young children; in patients with severe intractable asthma who are constantly taking adrenalin; in patients too ill or too feeble for skin tests; in those in whom severe constitutional reactions are feared and, finally, as a check upon the specificity of positive skin reactions under special conditions.

1. The test depends upon the possibility of sensitizing a local area of skin of a normal individual by the intracutaneous injection of the serum of an allergic individual carrying skin-sensitizing antibodies or allergins as in hay fever or asthma (Prausnitz-Kustner method of passive transfer of antibody). This test cannot be done if the allergic individual is syphilitic. Remove 5 cc. of blood from the allergic individual with aseptic precautions. Separate the serum and centrifuge if necessary to remove corpuscles. Keep in refrigerator. It may be preserved by the addition of 0.1 cc. of 5 per cent phenol per cc. It is advisable to culture for sterility.

2. With a sterile tuberculin syringe fitted with a No. 26 needle, inject 0.1 cc. of the serum *intracutaneously* into the forearm of a normal nonallergic individual. As a general rule 8 injections may be made in 2 rows of 4 each. Do not use senile skins; avoid sunburned areas. If foods are being tested the individual should abstain from these for 48 hours and especially in the case of eggs, fish and nuts. Ring each site of injected skin with ink or skin pencil.

3. Allow 48 and preferably 72 hours to elapse and reinject the *sensitized sites* with 0.02 cc. of sterile solutions of the allergens being tested; at the same time inject a similar amount of each into adjacent areas of nonsensitized skin as controls. Also

inject 0.02 cc. of normal saline solution into a sensitized area as an additional control.

4. Inspect both areas 10 minutes later. A reaction in a sensitized site with no reaction in the control site is positive. If both are negative it is likely that the serum had no antibodies for the allergen employed. If both are positive the individual has an allergy to the allergen and the test would have to be repeated with another individual.

### METHODS FOR CONDUCTING IMMUNOLOGIC SKIN TESTS

**Schick Test.**—1. The usual method is to inject 0.1 cc. of the diluted toxin *intracutaneously*, using the cleansed skin of the inner surface of the forearm below the elbow or the upper arm near the site of insertion of the deltoid muscle.

2. In the case of older children and adults, a similar injection of 0.1 cc. of the control fluid should be made either in the opposite arm, which is preferable, or several inches below the test site on the same arm. It is essential that the injections be given into the skin and not subcutaneously; otherwise, false negative reactions may occur. The presence of a *large white palpable wheal* indicates that the injection has been properly made.

3. Accuracy in the measurement of the amount of toxin injected may be insured by the use of a properly graduated tuberculin syringe, although a good Luer or Record syringe also is satisfactory. A 26-gauge  $\frac{1}{4}$  to  $\frac{1}{2}$  inch hypodermic needle is best adapted for intracutaneous injections. If the test is being performed on a group of individuals, it is permissible to use the same needle for all injections, providing it is wiped off with alcohol after each injection.

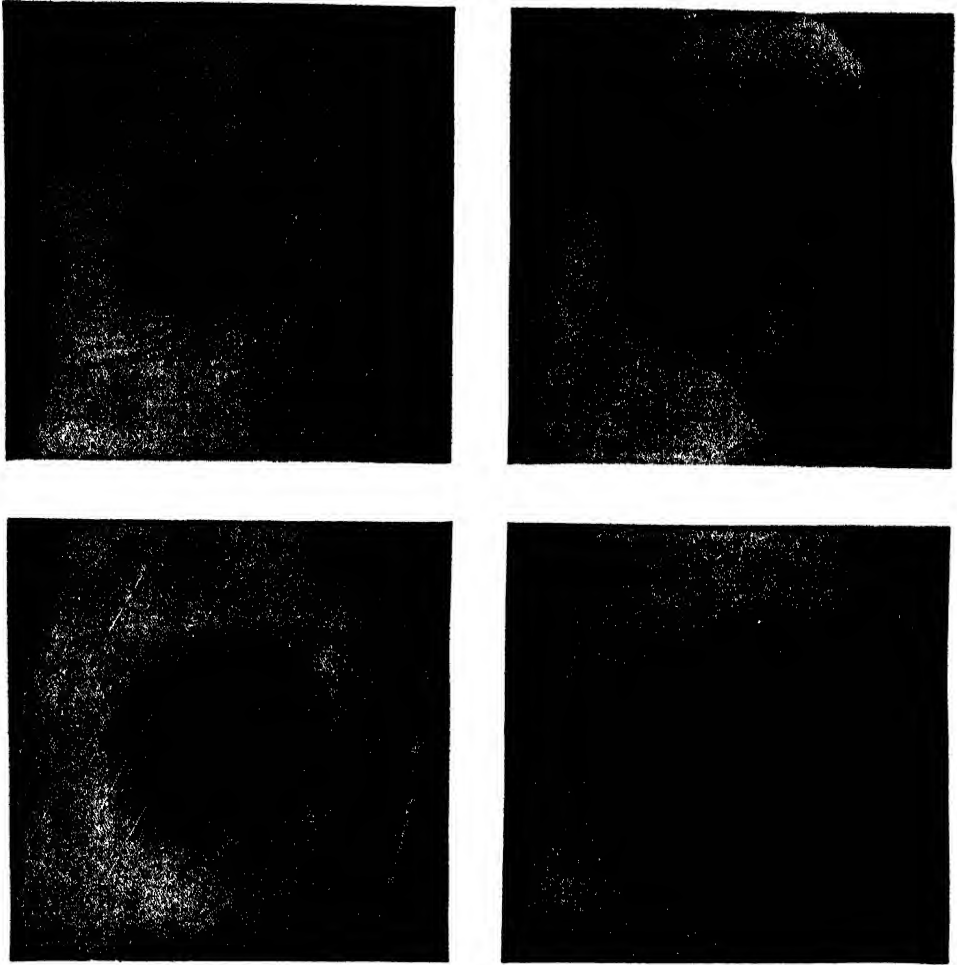
4. The reactions are best read and interpreted on the fifth to seventh days after the injection of toxin. True *positive reactions* (Plate XVII) may vary from a small area of erythema with slight edema about the size of a quarter (faintly positive) to that of a half dollar (definitely positive) or larger, with considerable edema (strongly positive).

5. *Pseudopositive reactions* (Plate XVII), which have the same significance as negative reactions, develop within 6 to 18 hours and reach their height in 36 to 48 hours, usually disappearing by the fourth or fifth days. They are characterized by a central area of dusky redness about the size of a dime with a secondary pale areola that shades off into the surrounding skin, with little or no edema.

6. *Combined true and pseudoreactions* (Plate XVII) have the same significance as true positive reactions. They are characterized by a central zone of dusky erythema with considerable edema along with a wide pale areola of erythema.

**Dick Test.**—1. This is exactly like that of the Schick test and consists of the *intracutaneous* injection of 0.1 cc. of Dick toxin carrying one skin test dose (one S.T.D.). The syringe and needle should not be sterilized with alcohol or other disinfectants, since retention of the latter may result in destruction of the toxin. Distilled water for boiling is preferable to tap water and as much of the water as possible should be expelled from the syringe and needle before use; this may be aided by ejecting a little toxin from the needle just before making the injection. As previously stated, rigid aseptic precautions are required to prevent any bacterial contamination that may result in falsely positive reactions. It will be noted, therefore, that the technic of this test is exacting.

## PLATE XVII

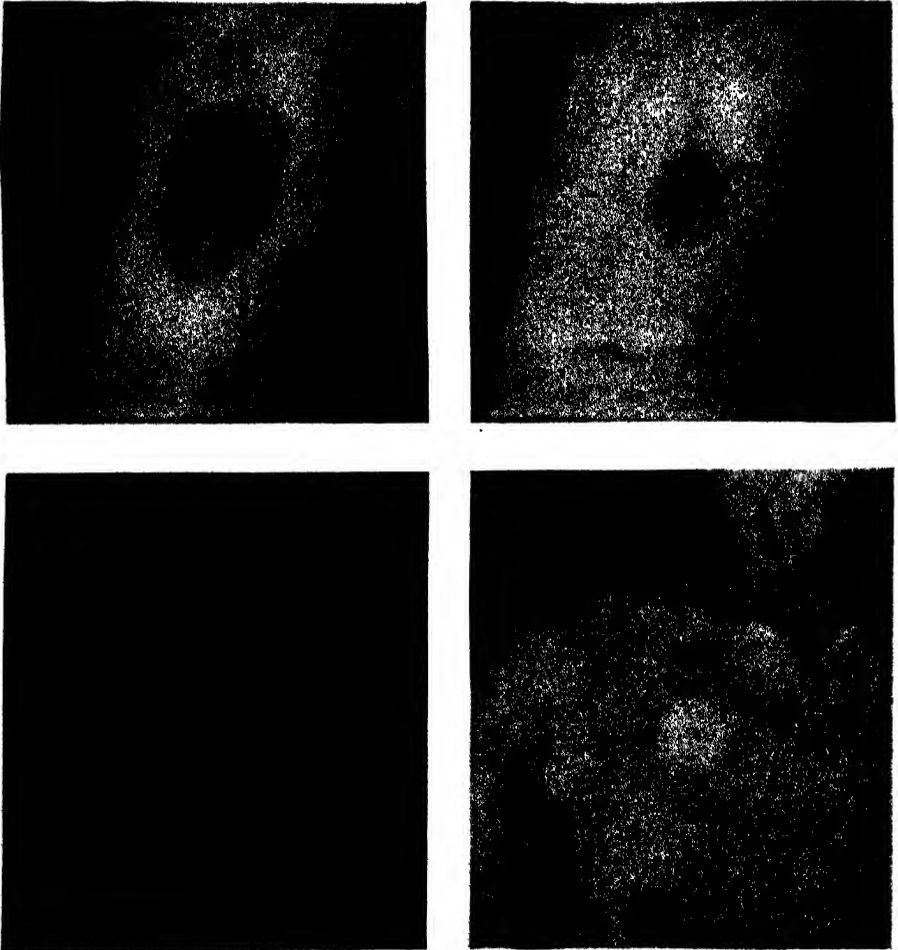


### POSITIVE SCHICK REACTIONS

*A*, positive reaction at end of 48 hours; *B*, positive reaction at end of 4 to 5 days; *C*, pseudopositive reaction; *D*, combined true and pseudopositive reaction.

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

## PLATE XVIII



### POSITIVE DICK AND SCHULTZ-CHARLTON BLANCHING REACTIONS

*A*, strongly positive Dick reaction at end of 24 hours; *B*, slightly positive Dick reaction at end of 24 hours; *C*, strongly positive Dick reaction in a Negro at end of 24 hours; *D*, Schultz-Charlton blanching reaction.

(From Kolmer, *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co., New York.)

2. Reactions should not be read earlier than 18 hours or later than 24 hours after the injection of toxin.

3. *Positive reactions* (Plate XVIII) are characterized entirely by erythema without induration or edema and may be faintly or slightly positive (between 10 and 20 mm.), moderately positive (between 20 and 30 mm.), strongly positive (between 30 and 40 mm.) or very strongly positive (over 40 mm.). The slightest erythema, no matter how faint, constitutes a positive reaction if it measures as much as 10 mm. in any direction.

4. *Falsely positive reactions* are sometimes due to infection of the skin; they usually do not suppurate but last longer than a true positive reaction and are often indurated. *Pseudoreactions* due to allergy are very infrequent.

**Schultz-Charlton Blanching Test in Scarlet Fever.**—This test has proven of clinical value as an aid in the differential diagnosis of scarlet fever rashes from clinically similar rashes occurring in rubella and the drug allergies (quinine, salicylates, etc.). It consists of the *intracutaneous* injection of 0.5 cc. of convalescent scarlet fever serum or 0.1 cc. of a potent scarlet fever antitoxin in the center of a large area where the rash is brightest, preferably on the abdomen or chest. The reaction is observed 18 to 24 hours later. A *positive reaction* consists of blanching of the rash in a zone surrounding the central red spot where the injection was made (Plate XVIII). The reading should be made while standing several feet from the patient. Rashes due to drug allergies are not blanched. The same is true in rubella (German measles) if the test is conducted with scarlet fever antitoxin. Convalescent scarlet fever serum may cause some blanching of nonscarlatinal rashes due to infectious diseases if it happens to contain specific antibodies for them. Consequently, the test is best conducted with standard scarlet fever antitoxin.

**Pneumococcus Antibody Test.**—This test, as described by Francis (*Jour. Exper. Med.* 57: 617, 1933), is conducted by injecting intradermally 0.1 cc. of a sterile protein-free solution carrying 0.01 mg. of the type-specific capsular polysaccharide. Positive reactions which develop within 15 to 30 minutes, occur at about the time of recovery and are apparently the result of interaction between antibody and the polysaccharide. Therefore they are indicative of the presence of adequate amounts of antibody and of good prognostic import. Negative reactions, however, are indicative of severe infections and the presence of inadequate amounts of antibody in the blood and tissues, with the need for the administration of adequate amounts of sulfadiazine and type-specific immune serum.

**Hemophilus Influenzae Antibody Test.**—*Hemophilus influenzae* belonging to type B is also known to produce a type-specific soluble polysaccharide. Recovery from severe infections with the bacillus, as in influenzal meningitis and pneumonia, is due largely to specific antibody supplemented by sulfonamide therapy. As shown by Alexander and her colleagues (*Jour. Pediat.*, 20: 673, 1942) whether or not adequate amounts of antibody are present in the blood may be determined by the capsular swelling or skin tests.

The skin test is conducted by injecting 0.1 cc. of a 1:5000 dilution of type B *H. influenzae* polysaccharide intracutaneously. A positive reaction, indicative of an excess of antibody in the patient, is of the immediate type developing within 5 to 10 minutes and remaining for approximately 30 minutes. It is characterized by a wheal with extrusion of pseudopods.

### METHODS FOR THE PREPARATION OF ALLERGENS

Prepared extracts of a very large number of different substances as pollens, hairs, dandruffs and feathers of the lower animals, various foods, silk, cotton, glue, orris root, etc., may be obtained from manufacturing laboratories suitable for the scratch or cutaneous test. These are usually in the form of powders or solutions.

Fluid extracts of allergenic substances may be obtained in the same manner suitable for intracutaneous tests as well as the allergenic oils of pollens, poison ivy, sumac, oak, etc, for the patch test. It is unnecessary, therefore, to include here the technic of their preparation.

Stock dust extracts may be likewise obtained from the same sources, but autogenous extracts prepared of house or industrial dusts to which the individual patient is exposed, are sometimes required. These, as well as extracts of foods or other materials, may be prepared as follows for intracutaneous tests and treatment by desensitization:

*Principles.*—1. The extract should contain sufficient of the active excitant or allergen to give positive skin reactions in sensitive individuals.

2. It should be prepared with the minimum of denaturing of the active allergen. This is probably of special importance with foods. In the case of foods eaten in the raw state it is preferable to prepare extracts of the raw material. In the case of cooked foods it is preferable to prepare the extracts after the usual amount and degree of cooking as it would appear possible for an allergic individual to give a negative reaction to an extract of raw food and a positive to an extract of the same food prepared after cooking; likewise, the reverse.

3. It should be sterile and nonirritating to the skin.

4. It should be of such strength that the test dose does not produce nonspecific reactions in normal nonallergic individuals.

*Method.*—1. Divide the food very finely and include the juices. The wet food may be extracted or it may be dried and pulverized. With dust (collected in a vacuum cleaner from the floors, rugs, draperies and mattress), remove match sticks, hairpins, etc.

2. Place the material in a flask and add 2 or 3 volumes of carbon tetrachloride. Stopper tightly and shake thoroughly. Place in incubator at 37° C. for 1 or 2 days. Decant and discard the carbon tetrachloride and add 2 or 3 volumes of water-free ether. Stopper tightly, shake and keep at room temperature for 1 or 2 days with frequent shakings. These measures are for the removal of fats and coloring matter.

3. Filter through paper and allow the material to dry on the paper overnight.

4. Transfer the material to a flask and add an equal volume of one of the following extracting fluids:

#### COCA'S EXTRACTING FLUID

Sodium chloride .....	4.0 gm.
Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) .....	1.4 gm.
Phenol (5 per cent) .....	40.0 cc.
Distilled water to make .....	500.0 cc.

When 2 or 3 drops of a 1 per cent alcoholic solution of phenolphthalein are added to 5 cc. it should remain colorless; to insure this state Coca advises passing carbon dioxide through the fluid before use.

## BUFFERED SALINE SOLUTION

Sodium chloride .....	50.0 gm.
Potass. dihydrogen phosphate .....	3.63 gm.
Disodium hydrogen phosphate .....	14.31 gm.
Distilled water .....	1000.0 cc.

Mix equal parts of this solution with 4 per cent solution of phenol. For use dilute 1 part with 4 parts of sterile distilled water.

5. The Coca fluid is advised for the preparation of extracts of pollens and dusts; the buffered saline for all other extracts. The latter is also used for preparing dilutions of extracts.

6. Cover with a thin layer of tuluol to inhibit bacterial activity and extract in the incubator for 48 hours.

7. Filter through paper, gently squeezing the material. The filtrate may be concentrated by evaporation in a large dish exposed to an electric fan while carbon dioxide is bubbled through or by ultrafiltration through cellophane.

8. Pass the filtrate through a sterile Berkefeld, Mandler or Seitz filter. It is advisable but not necessary to standardize by determining the total nitrogen by the Kjeldahl method or the protein nitrogen by the phosphotungstic acid precipitation method. As a general rule satisfactory standardization may be accomplished by testing normal individuals with intracutaneous injections of 1:5, 1:10, 1:100, etc., to determine the lowest dilution giving a completely negative reaction.

9. Culture for sterility by placing 2 or 3 cc. in a flask of 100 to 200 cc. of broth (pH 7.2) and incubate at least 5 days.

10. Keep the extract in a refrigerator where it usually maintains allergenic activity for about 1 year. If in bulk, cover with a thin layer of toluol.

*Notes.*—1. Hairs and dandruffs are apt to be particularly greasy and require thorough de-fatting. Extract with buffered saline solution.

2. Feathers should not have been previously washed or immersed in water.

3. With cereals cover the extracting fluid with toluol. Use minimum amount of extracting fluid. Dialysis of the extract aids in removing viscosity and makes filtration easier (also applies to orris root).

4. Nuts, seeds and beans should not be roasted; grind in a coffee grinder and thoroughly remove the oils with ether before extracting. Dialysis aids in removing irritating substances.

*Method for Milk (Coca).*—1. Remove all fat from 500 cc. by centrifuging.

2. Add 2.5 cc. of 1 per cent rennin and place in a water bath at 37° C. for ½ hour without stirring.

3. Remove the precipitated casein by straining through a sterile towel.

4. Add 7 cc. of a saturated solution of sodium carbonate to the filtrate.

5. Filter through a sterile Berkefeld, Mandler or Seitz filter and culture for sterility.

*Method for Eggs.*—Dilute whole egg white 1:10 and 1:100 with the buffered saline solution. Sterilize under tuluol for 3 or more days. Culture for sterility.



### TESTS FOR SERUM ALLERGY

These are frequently required before the administration of immune sera for the prophylaxis and treatment of disease, to determine if the patient is hypersensitive.

1. If the patient is an asthmatic and especially if suspected of being sensitive to horse serum, a scratch or *cutaneous* test should be first conducted with a drop of normal horse serum. A positive reaction indicates such a high degree of sensitiveness that serum should not be given at all or only by very special methods.

2. If the history is negative for asthma, or if this test gives a negative reaction, inject *intracutaneously* 0.02 cc. of sterile normal horse serum (0.2 cc. of 1:10 dilution). A control injection of sterile saline solution is advisable. Read reactions in about 10 minutes. A positive reaction indicates that if serum is given precautions are required and especially if administered intravenously. The test is sometimes conducted with the immune serum for administration. This is not advisable because the antibodies in the serum may produce an immediate erythematous and edematous reaction (known as the *E-E reaction*) which may be mistaken for a positive allergic reaction (Foshey, L.: *Jour. Allergy*, 1935, 6, 360).

3. An *ophthalmic* test may be conducted by placing a drop of 1:10 dilution normal horse serum in the lower conjunctival sac. The test cannot be conducted in the case of crying children. Wait about 10 minutes. A positive reaction is shown by erythema, lacrimation and some itching. It is not as reliable as the intracutaneous test.

### METHODS FOR CONDUCTING ALLERGIC SKIN TESTS IN BACTERIAL, MYCOTIC, VIRAL AND PARASITIC DISEASES

**Tuberculosis.**—The cutaneous or scratch test of von Pirquet, employing old tuberculin, is highly specific but less sensitive than the intracutaneous test of Mantoux. Therefore, the latter is preferred and may be conducted with old tuberculin (O.T.) or the purified protein derivative (P.P.D.) of Seibert which is preferred. If old tuberculin is employed, 0.1 cc. of a 1:10,000 dilution (0.01 mg.) is injected intracutaneously but if a negative reaction is observed a second injection of 0.1 cc. of a 1:100 dilution (1 mg.) should be made. If P.P.D. is employed, a tablet of the first test strength (0.00002 mg.) is dissolved in the vial of diluent supplied and the injection given intracutaneously, but if a negative reaction is observed, a second injection is given employing a solution of a tablet of the second strength dose (0.005 mg.).

Reactions should be read 48 hours later. Negative reactions show no edema with a very slight degree of erythema. Positive reactions are characterized by edema with erythema varying in degree from  $\pm$  (erythema less than 5 mm. in diameter with only a trace of edema) to  $++++$  (marked erythema, edema and an area of necrosis).

**Brucellosis.**—A skin test of aid in the diagnosis of this disease may be conducted by the intracutaneous injection of 0.1 cc. of *brucellergen* (Huddleson) composed of the protein nucleinate fraction of *Brucella*, after the lipid fraction has been removed. Reactions are best read 48 hours later and recorded in the same manner as tuberculin reactions.

*Positive reactions* are characterized by circumscribed areas of erythema, induration and edema, sometimes accompanied by systemic reactions, which may persist for a week or longer. Frequently they do not occur until the disease is well developed with

positive agglutination reactions. But in the absence of the latter, they are usually indicative of the disease, providing there is a markedly positive opsonocytophagic reaction of the blood at the same time. Positive agglutination, brucellergen and opsonocytophagic reactions are practically conclusive evidence of infection. As in tuberculosis, however, anywhere from 6 to 10 per cent positive reactions may occur in the absence of clinically recognizable undulant fever, apparently due to minor infections, and especially among those who, like veterinarians and laboratory workers, are particularly exposed to infection, and those accustomed to the use of raw milk. Allergic sensitization also tends to persist with positive reactions occurring during convalescence and for long periods of time after apparent recovery probably due to persistent infection. *Negative* reactions indicate the absence of allergic sensitization but not necessarily the absence of infection since they may occur in individuals with brucellosis presenting such definite and conclusive evidences of infection as repeatedly positive blood cultures.

**Tularemia.**—Allergic sensitization to *Bact. tularensis* is apparently acquired early in the course of tularemia. For this reason skin tests conducted by the intracutaneous injection of killed suspensions of the organism (Foshay) are sometimes of value in early diagnosis, as positive reactions are stated to occur on the third or fourth days of the disease. As a general rule, however, agglutination tests are preferred. Negative reactions do not exclude the disease. Since the allergy usually persists for years after recovery, positive reactions do not necessarily mean active disease. When they are observed, however, in the presence of suspicious lesions, the latter is usually the proper interpretation. Apparently cross-reactions with suspensions of *Br. abortus* and *Br. melitensis* have not been observed, although they share a common antigenic constituent with *Bact. tularensis*.

**Chancroid.**—The antigen formerly used was prepared by diluting pus aspirated from buboes with saline solution and sterilizing with heat. At the present time it is prepared of cultures of *B. ducreyi* suspended in saline solution to carry about one billion per cubic centimeter and heated for 30 minutes at 60° C.

The test is conducted by the intradermal injection of 0.1 cc. and the reaction read after 72 hours. It is stated that positive reactions occur between 8 and 15 days after the appearance of the local lesion. About 95 per cent of cases of chancroid give positive reactions. Allergic sensitization apparently endures for many years and possibly for the balance of life. Consequently, this may account for the positive reactions observed in some cases of lymphopathia venereum, granuloma inguinale and normal individuals in whom chancroidal infection was present at some time in the past.

**Tests for Bacterial Allergy in Relation to Vaccine Therapy.**—1. Cultures are made on Petri plates of hormone blood agar and the different bacteria isolated and identified.

2. Pure cultures of these are grown on appropriate solid media for 24 to 48 hours and removed with sufficient saline solution to give dense suspensions of at least 2,000,000,000 per cc. Or the bacteria may be grown in a fluid medium and secured by centrifugation. If any medium has been carried into the suspension it is filtered through sterile paper and the bacteria are washed once with saline solution containing 0.5 per cent phenol before being resuspended in the saline solution.

3. Each suspension is heated in a water bath at 60° C. for 1 hour, cultured for sterility, and preserved with sufficient phenol to make 0.5 per cent.

4. For the skin tests a small amount of each suspension is diluted with sterile saline solution to give approximately 500,000,000 per cc.; 0.05 cc. of each is injected intracutaneously. A control injection of saline solution is made at the same time.

5. The reactions are read approximately 1 and 24 hours later.

6. Those bacteria causing positive reactions are then incorporated into an autogenous vaccine prepared from the stock suspensions.

**Dermatophytoses and Dermatophytids.**—These tests are conducted by the intradermal injection of *trichophytin*. Various preparations are commercially available. The test dose is usually 0.1 cc. of a dilution (usually 1:30 or 1:100) capable of producing reactions in sensitive individuals. The syringe and needle should be freshly sterilized by boiling. The trichophytin should be sterile. The readings should be made after 10 to 15 minutes (for an immediate wheal reaction which is especially apt to occur in infections with *T. purpureum*), after 48 hours (for the usual eczematous reaction) and again at the end of 1 week. A control injection of sterile broth is advisable. Strongly positive reactions should lead to conservative methods of treatment since the prognosis is favorable; when there is an exudative dermatitis the use of roentgen rays may be considered. Temporary falsely negative reactions may occur during some of the acute infectious diseases. Patch tests have also been employed but are not as satisfactory as intradermal tests.

**Coccidioidomycosis.**—Dickson has found intradermal injections of *coccidioidin* prepared of cultures of *C. immitis* of value in the diagnosis of coccidioidomycosis. The reaction is stated to be highly specific. But since sensitization may persist for long periods of time after recovery from primary infections of the lungs, positive reactions are not necessarily diagnostic of lesions of the skin suspected of being due to coccidioidomycoses. In conducting the test the needles and syringes should not have been used for similar tests with trichophytin or other antigens.

**Lymphopathia Venereum.**—As originally shown by Frei, a skin test has proven highly specific and of great value in diagnosis, conducted by the intracutaneous injection of 0.1 cc. of a 1:10 dilution of sterilized pus removed by aspiration from an unruptured inguinal bubo. The reaction is read 48 to 72 hours later. A positive reaction consists of an inflammatory papule with erythema and is stated to occur in 95 per cent of cases with buboes, or 90 per cent with ulcerative lesions. Negative reactions, however, may occur in the early stages of the disease. Unfortunately, nonspecific reactions occur and may be as high as 30 per cent. These reduce the practical value of the test as it is difficult to use a control antigen.

The virus has been successfully cultivated by the method of Rake, McKee and Shaffer in the egg yolk sac of the developing chick embryo and by differential centrifugation may be obtained in a high state of purity. Suspensions in 0.1 per cent formalin are slightly hazy and almost colorless solutions with so little extraneous matter that intracutaneous injections of 0.1 cc. are not nearly as likely to produce nonspecific reactions as suspensions of sterilized pus or mouse brain. This type of antigen, therefore, is strongly recommended and is commercially available. Positive reactions are characterized by reddish papules 6 mm. or more in diameter, surrounded by a fainter areola of erythema of varying size. The papule is the important part of the reaction and should be measured with a millimeter scale. Strongly positive reactions may show pustules or vesicles. Reactions gradually regress leaving a faintly pigmented area with or without a slight scar.

**Echinococcus Disease.**—An intracutaneous test has proven of value in the diagnosis of this disease although its clinical application may be hampered by difficulties experienced in obtaining a suitable antigen for its conduct. The latter is generally prepared of pooled sterile hydatid fluids obtained by puncture of unilocular hydatid cysts of sheep, pigs, oxen or human beings. After filtration and culture for sterility it is placed in ampules which, when kept in a refrigerator, are ready for use up to 6 months. With antigen supplied by the National Institute of Health, the technic and reactions are as follows:

The usual test dose is 0.01 cc. of 1:10,000 dilution. It is advisable to include a control consisting of the intracutaneous injection of 0.01 cc. of sterile saline solution. A positive reaction is of the immediate type, appearing usually within 15 to 20 minutes after the injection of the antigen. In rare cases there may be a delayed reaction which does not reach its height before 24 hours. It is characterized by the formation of a wheal with a diameter larger by 3 mm. or more than that of the control, with or without pseudopodia. The wheal is usually surrounded by a zone of hyperemia, but this is not as important as the size of the wheal and the presence of pseudopodia. The test is particularly useful preoperatively. In postoperative cases positive reactions may be observed over long periods of time. Positive reactions, however, are not absolutely indicative of the disease, since falsely positive or pseudoreactions may occur from hypersensitiveness to sheep protein in the antigen, from trauma, and in other cestode infestments. A negative reaction is valuable, but not conclusive, evidence for excluding hydatid disease.

**Trichinosis.**—An intradermal test has proven of value in the diagnosis of infestation with *Trichinella spiralis* and especially in mild cases with only vague symptoms. The antigen may be prepared according to the method described by McNaught, Beard and Myers (*Am. Jour. Clin. Path.*, 11: 195, 1941). With this antigen the test dose is 0.1 cc. of a 1:10,000 dilution injected intradermally. A control injection of 0.1 cc. of sterile buffered saline solution is given at the same time.

During the second or third weeks of the disease an immediate reaction consisting of a wheal with a zone of hyperemia is commonly observed. During the first few days of the disease, and especially in long-standing quiescent cases, a delayed reaction usually occurs, reaching a maximum size of 1 to 3 cm. in 20 to 24 hours and resembling a mild tuberculin reaction which then subsides.

**Filariasis.**—Intradermal tests conducted with antigens prepared of canine *Dirofilaria immitis* have proven of clinical value since positive reactions occur in about 90 per cent of cases of filariasis due to infestation with *Wuchereria bancrofti*. The reactions are usually of the immediate type but delayed reactions characterized by marked edema resembling Calabar swellings may occur. Similar results have been observed in loiasis and onchocerciasis with this antigen as well as with antigen prepared of *Onchocerca volvulus* so that the reactions are group-specific for filariae. Positive cutaneous reactions have been reported in dracunculiasis many years after recovery.

**Schistosomiasis.**—Intradermal tests conducted with cercarial antigens prepared of snails infested with *Schistosoma spindale* or *S. bovis* give a high percentage of positive reactions in schistosomiasis of human beings due to *S. hematobium* and in a relatively small percentage of pseudopositive reactions in noninfested individuals due to hypersensitiveness to the proteins of the snail. Similar results have been re-

ported with cercarial antigens prepared of *S. mansoni* so that the reactions are of a group character. Intracutaneous tests, however, possess some diagnostic value and antigens prepared of *S. japonicum* have been particularly recommended for field work in endemic areas, since positive reactions occur but rarely in noninfested individuals. Undoubtedly positive reactions continue to occur over long periods of time after apparent recovery.

**Leishmaniasis.**—Intradermal tests conducted with phenolized saline suspensions of dead *Leishmania tropica* almost invariably give positive reactions in oriental sore but may also give weakly positive reactions in about 10 per cent of noninfected individuals.

**Diodrast Allergy.**—Since systemic reactions may follow the intravenous administration of diodrast for excretory pyelography, which are frequently disturbing and occasionally serious, Naterman and Robins have stated that natural allergy to this substance may be one cause for their occurrence. Consequently they advise that an intradermal test, consisting of the injection of 0.05 cc., should be done on all patients before diodrast is injected intravenously. A strongly positive reaction is characterized by the development of a wheal larger than 15 mm.; in such cases intravenous injections may give serious reactions and require that precautionary measures be taken.

# CHEMICAL METHODS

## METHODS OF COLORIMETRY

**Principles.**—1. Most of the biological constituents occur in quantities too minute to be determined gravimetrically with the amount of sample available. Under the conditions either volumetric or colorimetric methods are employed. Of these, the latter is most often employed because of its speed and simplicity.

2. Colorimetry depends upon the quantitative comparison of colors developed in unknown solutions with those of solutions of known concentration. It is based on Beer's law which states that the light transmitted by a colored medium is inversely proportional to the concentration. In practice this linear proportionality is limited to a small range of concentration of a particular substance so that several comparison standards covering a wider range, are customarily used to attain greater accuracy. The standard may be composed of a solution of the substance to be analyzed and the colors developed in both unknown and standard simultaneously or they may be "permanent" colored solutions corresponding to known concentrations. Theoretically, the former type is more desirable since slight variations of temperature, time of standing, etc., will occur in both standard and unknown. However, "artificial" standards are often employed and at times are more desirable, particularly if the standard solutions have poor keeping qualities.

## COLORIMETRIC METHODS

The types of apparatus commonly employed in colorimetry are as follows: 1. *Visual*: (a) block comparator; (b) dilution colorimeter and (c) comparison colorimeter. 2. *Photoelectric*: (a) single cell and (b) twin-cell.

**Block Comparators.**—The block or rack comparator is used most frequently in the determination of certain substances in the urine in which accuracy may be sacrificed for simplicity and speed. They are adapted for fixed types of examinations and employ series of graded, permanent, standards against which the color or turbidity of the unknowns are compared as, for example, in the phenolsulfonephthalein kidney function test employing the Dunning colorimeter (Fig. 95).

**Dilution Colorimeters.**—The dilution comparator employs a single fixed standard and the unknown is diluted until it matches this standard. The Sahli hemometer (Fig. 39) is of this type and, although more accurate than the block comparator, is rather inflexible in that it is adapted for specific determinations only.

**Comparison Colorimeter.**—The comparison colorimeter is commonly employed because it is capable of considerable accuracy as well as flexibility. In general it consists of an arrangement whereby the thickness of the colored solution may be varied in either or both the standard and unknown so that color intensities may be varied

over a considerable range. One form of this type is the Duboscq colorimeter shown in Figure 314.

### DUBOSCQ METHOD OF COLORIMETRY

1. The solutions to be compared are placed in glass cups which are raised by means of rack and pinion until the lower ends of the clear glass plungers are immersed in the fluid, the excess of fluid rising between the plungers and the walls of the cups. By raising or lowering the cups the layer of fluid between the lower ends of the plungers and the bottom of the cups may be made of any desired thickness and the thickness of each is indicated by a scale placed in a convenient position. Beneath the cups is a mirror which reflects light up through the cups and the long axis of the

plungers into a series of prisms. These reflect the light from the two cups into a single field which is viewed by an eye lens. Each lateral half of the field receives its light through one of the cups. The raising or lowering of the cups, by diminishing or increasing the thickness of the layer of fluid through which the light passes, diminishes or increases the depth of color of the corresponding half of the field.

2. Adjustment of the scales should be tested before an instrument is used. Raise the cups gently until their bottoms are in contact with the plungers. If the reading is not exactly zero on each side, the scales, which are movable upon most instruments, must be brought to accurate adjustment. In the absence of an adjustable scale, a

correction must be made in the readings on each side, sufficient to indicate the true depth of liquid between the bottoms of the plungers and the top surfaces of the cup bottoms.

3. Test for the equal transmission of light through both sides of the light field by placing the same colored solution in both cups, taking care that they are not filled so much that displacement of fluid by the plungers causes the cups to overflow. Raise the cups until both plungers are immersed, and are set to have the same depth. So place the colorimeter with respect to the light source that the same amount of light is reflected up through each cup. Either daylight or artificial light, filtered through daylight glass, may be used. In neither case should the light be too strong, or the readings will be less accurate. If both halves of the field do not match exactly, the position of the instrument may be shifted slightly in an attempt to make them match. If a match is still not obtained, something differs in the optics of the two sides. The most common source of error in this respect is bubbles under the plungers.

4. After the colorimeter is correctly adjusted with respect to the light source, it should not be moved during the determinations. Empty the left hand cup and rinse it and the plunger with the standard solution, partly fill the cup, and raise it until the plunger is immersed, avoiding bubbles under the latter. Set the left cup at a convenient

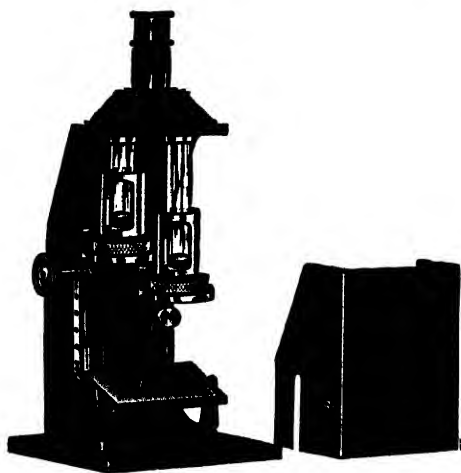


FIG. 314.—SMALL DUBOSCQ COLORIMETER

depth, usually 10, 15 or 20 mm., depending on the concentration, and adjust the depth of the right cup containing the unknown, until the 2 halves of the field have the same color intensity. Read and record the depth. For maximum accuracy make a number of readings and average the results, but guard against eye fatigue. Concentrations of the two solutions are inversely proportional to their depth when the color intensities are equal, assuming that Beer's law is followed. This relationship may be expressed by the general formula:

$$\text{Concentration of unknown} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{concentration of standard}$$

5. To express the results in relation to the concentration per 100 cc. of original sample, the basic formula must be modified by two further factors, namely, (a) the proportion of the quantity of the unknown used in the determination to 100 cc. and (b) the relative volumes to which the standard and unknown have been diluted for color comparison. These conditions vary with different determinations and the calculations are given under the separate methods. However, the following example illustrates the method employed. In the determination of nonprotein nitrogen the standard contains 0.15 mg. of nitrogen in a final volume of 50 cc. and the unknown, equivalent to 0.5 cc. of blood (5 cc. of 1:10 dilution) is also in a final volume of 50 cc. If the standard is set in the left-hand cup of the colorimeter to a depth of 20 mm. and the unknown in the right-hand cup reads 24, then the *concentration of unknown in mg. per 100 cc. of blood* is  $\frac{20}{24} \times 0.15 \times \frac{50}{50} \times \frac{100}{0.5} = \frac{20}{24} \times 30$ , or  $\frac{600}{24}$ .

Frequently, the calculation may be simplified by placing the *unknown* instead of the standard in the left-hand cup which is then set at a depth selected according to the concentration of the *standard* so that its concentration is an even multiple of the setting. Thus in the nonprotein nitrogen determination, since the standard contains 0.15 mg. in 50 cc. (the same volume as the unknown), if the unknown is set at 15 mm. then, using the same general formula, the concentration of the unknown per 100 cc. =  $\frac{\text{reading of standard}}{15} \times 0.15 \times 200$ , which reduces to  $2 \times \text{reading of standard}$ .

6. Results in colorimetric work are always most accurate when the unknown and the standard have nearly the same depth of color.

7. Where light absorption after a color-developing reaction is most intense in a particular portion of the spectrum, it is sometimes advantageous to employ light filters transmitting only this part of the spectrum. Trouble caused by the absorption of light in other parts of the spectrum by foreign substances may thus be eliminated.

## PHOTOELECTRIC METHODS OF COLORIMETRY

**Principles.**—1. The photoelectric colorimeter is based on the well known property of light to create an electric potential in a photoelectric cell. Since the current output of the cell depends upon the intensity of light reaching it, it follows that not only will the cell be affected by colored solutions interposed between it and the light source, but also by turbid solutions as well. The light intensity is measured by means of an electrical circuit which includes a resistance and a galvanometer. The indicator



of the galvanometer swings over a scale indicating in arbitrary units the amount of current generated, or the galvanometer swing is controlled by a variable resistance and the amount of resistance is measured on an arbitrary scale.

2. For strictly monochromatic light, the ratio of the intensity of light leaving a colored solution to that entering it is a logarithmic function of the concentration. In the commonly used instruments, monochromatic light is approximated by the use of light filters, covering a narrow band of wave lengths, and when the appropriate light filters are used it is usually found that Beer's law for colorimetric procedure is valid over a much wider range than in the visual colorimeter.

3. If the photoelectric colorimeter is provided with a logarithmic scale the readings plotted against concentration on cross section paper will fall on a straight line. If the instrument is equipped with a unit scale, readings plotted against concentrations on semi-logarithmic paper will form a straight line.

4. In the first type of instrument the concentration of unknown solutions is obtained directly by multiplying the scale reading by the proper factor predetermined from a standard. In the latter type of instrument calibration curves must first be plotted on semi-logarithmic paper for each procedure and, assuming constant conditions, values of unknown may then be interpolated from these graphs.



FIG. 315.—KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER

5. Since the photoelectric cell is exceedingly sensitive to small light fluctuations, the *light source must be constant*. In the single cell type this is usually attained by employing a constant voltage regulator, if used on the house current, or a storage battery. In the twin cell type of instrument, line voltage fluctuations which would cause variations in the intensity of the light source are automatically compensated by the use of the double photoelectric cells, which once balanced, are equally affected by the light source.

**Single Cell Type.**—The Evelyn photoelectric colorimeter is of this type. It employs a single, barrier-layer type of cell and a light source of low intensity and voltage

(storage battery) to avoid cell fatigue. The galvanometer swings are measured directly on an arbitrary unit scale and the comparison cells are calibrated test tubes. It is capable of high precision, being designed for constant reproducibility of results using semi-logarithm graphs as indicated for this type of instrument.

**Twin Cell Type.**—The Klett-Summerson photoelectric colorimeter (Fig. 315) is of this type. It is a self-contained unit which, because of the inherent compensating effect of the double photoelectric cell null-point principle, can be attached to any convenient source of alternating or direct current. Galvanometer swing is measured by a slide wire resistance with an attached logarithmic scale. Calibrated test tubes are used for comparison cells and the concentration of the unknown is calculated directly from the scale reading by the use of a predetermined factor obtained from a standard which has been put through the same procedure. Or, as in visual colorimetry, the standard and unknown may be prepared for each determination and readings made of both.

The colorimeter may be placed in any convenient position in the laboratory that is free from extraneous mechanical vibration and the presence of corrosive fumes. There should not be a strong light overhead nor should direct sunlight be allowed to strike the colorimeter tube.

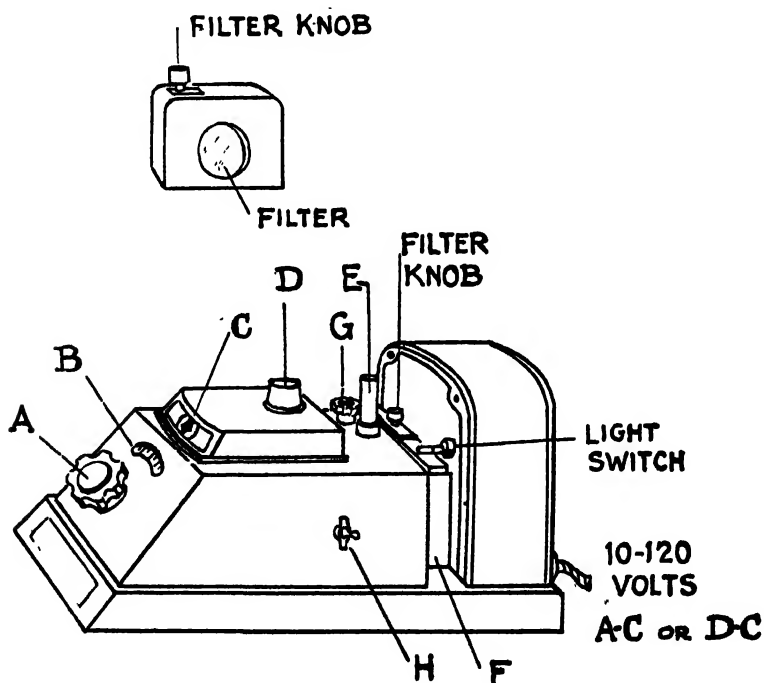


FIG. 316.—DIAGRAM OF KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER

The Klett-Summerson instrument is operated as follows: 1. With the light switch off (see Fig. 316) attach the electric cord to any convenient outlet. Be sure that a light filter (F) is in place in the space provided for it between the lamp housing and the instrument proper. Give the short-circuit switch (H) a quarter turn so that the

galvanometer is connected in the circuit. By turning the small knob (D) on top of the instrument, adjust the pointer (C) so that it coincides exactly with the line on the blank scale. These adjustments are made when the instrument is first placed and with the light off; the pointer setting usually remains unchanged for the same position of the instrument, although it is well to note that this is so each time before the lamp is turned on. Turn the scale by means of the large knob (A) until the scale reading at B is zero.

2. Insert a colorimeter tube (E), containing about 6 cc. of distilled water, in the instrument and turn on the lamp switch. It will now be found that switching on the light has caused the pointer (C) to move away from the line. Turn the zero adjustment knob (G) one way or the other until the pointer is again brought back to the line. Permit the lamp to burn for a few minutes to allow the instrument to reach equilibrium and again check the position of the pointer, bringing it back to the line with knob G if it has moved. This is called setting the zero, or zero reading.

3. To read an unknown solution replace the distilled water tube with a colorimeter tube containing the unknown solution. This deflects the pointer and this time it is brought back to coincide with the line on the blank scale by turning knob A. Turning this knob moves a contact over a circular slide wire resistance to which a logarithmic scale is attached. The scale reading is the "reading of the unknown or standard." The concentration of unknown is calculated by multiplying this scale reading (corrected for the reagent blank) by a factor obtained by dividing a known concentration of this same substance by the scale reading obtained for this concentration. The blank reading is obtained by running through the complete colorimetric procedure on a sample of distilled water. Both the blank and standard solutions should be run through the particular procedure in duplicate or triplicate and the values noted. Since ordinarily they remain constant for the same set of reagents, these values need not be determined each time an unknown is processed. However, an occasional check on the constancy of the figures is advisable.

4. The choice of light filters depends upon the particular analytical procedure being used. In general, the filter to be selected is the one which has a spectral transmission opposite to that of the solution being measured, *i.e.*, the filter which transmits the most light over the range where the solution absorbs most light. In this way maximum sensitivity is obtained. In some procedures, however, the colors produced (as in the unmodified Folin-Wu blood sugar method) would exceed the capacity of the instrument if such a filter were used, so that a filter is selected which absorbs in the same light range, thus affording a more satisfactory relationship between scale reading and concentration. It is also advisable at times to employ higher dilutions of sample than is customary with visual colorimetry in order to keep the resultant colors within practical range of the electric instrument.

5. Photoelectric colorimeter tubes, as supplied by the makers of the instrument, are calibrated for equal light transmission if they are used with the identification mark facing the operator, since light transmission through a glass tube is variable around its circumference. For this reason not all test tubes of the correct diameter can be used as comparison tubes, but only those which will give the same readings with the same solution. The colorimeter tube must be kept scrupulously clean, both inside and out, and it is good practice, before inserting it in the colorimeter, to wipe off the

outer surface to free it of finger marks, etc., by means of a lintless cloth, and to hold the tube up to the light to be sure that the solution is free of air bubbles.

6. Since, other things being equal, the final results depend on the accuracy with which the colors are measured, the advantage of a photoelectric instrument is evident, especially when yellow or yellow-brown colors are to be matched.

7. Two or more analysts will always get the same results with the photoelectric instrument; such agreement is seldom possible with the visual type.

8. The time element is of considerably greater significance in photoelectric measurement. In many methods color development is a function of time, a factor which may not be fully appreciated in visual colorimetry where comparison is made with a standard undergoing the same progressive changes as the unknown. In photoelectric color comparison, each color is estimated separately and it is essential that such measurements be made at definite times within rather narrow limits.

### METHODS FOR USING THE ANALYTICAL BALANCE

The balance should be kept level in a place of even temperature. In manipulating the balance all movements should be carefully made so that only a click is heard when the beam is raised and lowered. Nothing should be placed on or removed from the balance unless the mechanical supports are in place to take the weight off the knife edges. Exception may be made, however, in the case of weights smaller than 1 gram, if the pan rests are raised. With beam and pan rests having separate controls, as is the case in a good balance, the left hand manipulating the wheel moving the beam also controls the pan rests by bringing pressure of the side of the little finger against the push button. For use in keeping the balance parts free from dust and chemicals there should be provided a camel's hair brush about 1 inch wide. Objects which are to be accurately weighed should not be touched with fingers but handled with crucible tongs or otherwise. The zero point should be checked before each important weighing.

**Determination of Equilibrium Point: Exact Method.**—The exact limits to which the pointer moves on either side of the center of the scale are observed for an unequal number of swings and the mean of each set noted; then counting the excursions to the left "minus" and those to the right "plus," half the algebraic sum, or the mean, is taken as the equilibrium point. The amplitude of swing should be about 5 divisions on either side of the center mark. The first swing should be neglected. It is good practice to read three swings to the left and two to the right. If, for example, readings are as follows:

<i>Divisions to Left</i>	<i>Divisions to Right</i>
— 5	+ 5.2
— 4.6	+ 4.8
— 4.3	
Sum — 13.9	+ 10
Mean — 4.63	+ 5

then the equilibrium point is  $\frac{-4.63 + 5}{2} = +0.18$  or 0.18 of a division to the right of the center.

**Weighing.**—Before weighings are started the true zero point should be determined by the above method. If the displacement of the zero point from the center of the scale is greater than one scale division, the balance should be readjusted. When weighing, one of two procedures can be adopted. By adjustment of the weights and rider through trial and error the equilibrium point can be made to coincide with the true zero point. In the other less time-consuming method the equilibrium point is determined when it is brought to within several divisions of the zero point and calculation is made to the zero point by the use of sensitivity values of the balance.

**Sensitivity of Balance.**—The sensitivity of a balance is defined as the number of scale divisions by which the zero point is displaced by an excess in weight of 1 milligram on one side or, otherwise stated, the weight required to cause 1 scale division displacement of the equilibrium point. With increasing loads the friction on the knife edges increases and the sensitivity diminishes, so that it is necessary to know the sensitivity for various loads, and determinations are made occasionally and values kept on a card in the balance case for the following loads: no load, 1, 5, 10, 20, 50 grams. In practice the sensitivity for a given load is found by determining the variation made in the equilibrium point when 1 milligram is added to the counterpoising weights, noting the number of divisions by which the equilibrium point is changed, and then calculating the weight which would produce a variation of 1 scale division. A good analytical balance of 200 grams capacity has a sensitivity of 0.0003 gram or less without load and 0.0004 gram or less with full load. As readings are easily made to at least one-quarter of a scale division, it is seen that such a balance is well capable of weighing with an accuracy of 0.0001 gram and manufacturers commonly specify such balances as having a "sensitivity" of 0.1 milligram.

**Weighing—Rapid Methods.**—The procedures above outlined are too time-consuming for most ordinary work and there are several shorter methods by which almost equally accurate weighings can be made. It is important, however, with most of these that the analyst know his balance, particularly the sensitivity with various loads and the loss of amplitude taking place with each successive swing, and any method used should be occasionally checked by the longer procedure:

With a balance where one can regulate the initial throw of the pointer almost at will, an easy method consists in determining the zero point in terms of one set of opposite consecutive extreme excursion points (*e.g.*, 4 divisions to the left and 3 divisions to the right) and then with load in place adjusting weights so that the pointer again moves to the same two points, or points *near by* such that the difference between the opposite consecutive excursions is the same as the difference between the zero point excursion values (*e.g.*, with excursions at the zero point of 4 to left and 3 to right, the loaded balance could be assumed to be in equilibrium at the zero point when the pointer makes excursions of 5 to the left and 4 to the right, or 5.3 to left and 4.3 to right, etc.).

If the zero point of the unloaded balance has been adjusted to coincide with that of the scale, in the final adjustment of weights the loaded balance can also be brought to this ideal zero point by causing the excursion of the pointer to the right to be a certain fraction of a division less than the preceding excursion to the left, this value being previously determined as one-half the loss in amplitude occurring during one complete cycle over approximately the same range of swing.

In the method of equal swings the balance is adjusted so that the zero point is

displaced to the right by half the amount of amplitude lost during one cycle. Then the weights are taken so that at the end of each swing to the right the pointer stops on the division corresponding to the starting point at the left. Any correction for known imperfect adjustment of zero point can be made by using the sensitivity values.

**Weights.**—Weights are always handled with the forceps. When the weighing is finished the empty places in the box and the rider position are noted and the weight recorded. This is confirmed by observation of the weights as they lie on the balance pan and may be reconfirmed by counting as weights are replaced in the box. Weights of good quality when new are accurate enough for ordinary work, but it may be desirable and for the most accurate work essential that the degree of error affecting the weights be known and be redetermined from time to time. Manufacturers of good weights allow a tolerance of  $\pm 0.2$  milligram in the 10-gram weights. In the calibration of weights, a standard 10-gram weight is best used. If a standard weight is not available, one of the 10-gram weights in the set can be chosen as a standard, and if all weighings are done with the one set of weights, no error in analytical work will be introduced by such arbitrary choice of standard.

One of the 10-milligram weights is assumed to be correct, and provisionally corrected values of all other weights are worked out in terms of it. By using the sensitivity of the balance, as explained above, the corrected weight of the other 10-milligram weight is found to be, for instance, 9.97 milligrams. If when the 20-milligram weight is placed on the left pan, and the two 10-milligram weights are on the right pan, the correction is found from the shift in the zero point to be  $+ 0.07$  milligram, the weight of the 20-milligram weight is recorded as  $10.000 + 9.97 + 0.07 = 20.04$  milligrams. Thus, provisionally calibrated small weights and the rider (also calibrated against the 10-milligram weight) are combined to weigh the next larger one. When all provisionally correct weights are worked out, these values are multiplied by a factor which makes the weight of the chosen standard 10-gram weight exactly 10.0000. For instance, if after calibration of all weights on the assumption that the 10-milligram weight is correct, the standard 10-gram weight is found to weigh 10.0032 grams, all values should be multiplied by the fraction

$$\frac{10.0000}{10.0032}, \text{ or } \frac{1}{1.00032}$$

**Accessories.**—In the so-called "chainomatic" balance final weight additions from 50 to 0.1 milligrams are made by simple adjustment in the length of a movable gold chain and this makes for more rapid and convenient weighing than is possible with the rider system. Weighing accessories consist of a spatula, a pair of matched watch glasses, glazed paper squares, a pair of crucible tongs, weighing bottles of several sizes, a large and a small camel's hair brush. A large feather cut to the shape of a flag on a staff is useful for transferring dry powders. When weighing is preceded by an ignition or hot drying operation, the object should be put in a desiccator while still warm, and after cooling there for twenty minutes or longer the weighing may be made. All objects must be at room temperature when weighed.

# METHODS FOR THE PREPARATION OF STANDARD VOLUMETRIC SOLUTIONS

*All solutions, unless otherwise specified, are to be prepared of C.P. grades of chemicals. The word water always implies distilled water.*

Normal and fractional normal volumetric solutions are not very stable, therefore they must be checked by titration if they have been standing for some time. Normal solutions may retain their strength for approximately five or six months; weaker solutions are less stable. Though a fractional normal solution can be made from a normal by dilution it is always a safer procedure to check the finished product by titrating it with a fractional normal solution known to be correct.

## SULPHURIC ACID SOLUTIONS

**Standard Normal Sulphuric Acid.**—*Principle.*—The strength of a solution of sulphuric acid, slightly stronger than normal, is determined by titration against a known amount of sodium carbonate. It is then diluted to exactly normal and the dilution confirmed by titration.

**Reagents.**—*Sulphuric acid*, Sp. Gr. 1.84.—*Methyl Orange.*—Dissolve 0.1 gram of methyl orange in water and dilute to 100 cc.

**Normal Sodium Carbonate Solution.**—Dry the sodium carbonate in an oven at 105° C. for 3 to 4 hours. Weigh on an analytical balance 5.300 grams; transfer quantitatively to a 100 cc. volumetric flask; dissolve in water and dilute to the graduation with water.

**Procedure.**—1. In a 2 liter beaker containing about 1100 cc. water, slowly add about 33 cc. sulphuric acid while stirring. Cool and transfer to a 2 liter glass-stoppered bottle. Mix thoroughly. Fill a 25 cc. buret.

2. Into a casserole pipet accurately 20 cc. of the normal carbonate solution. Add 2 drops methyl orange reagent. Titrate with the acid solution from the buret until a faint pink color remains on stirring. Read the amount of acid solution required from the buret. Repeat the titration with another 20 cc. of the carbonate. They should check within 0.1 cc.

3. Calculate the dilution as follows:

$N$  = average of titrations in cc.

$W$  = cc. of water to be added to the liter volumetric flask

$W = 50 (20 - N)$

4. Place  $W$  cc. of water in liter flask; add the sulphuric acid solution to the mark. Mix thoroughly. Place some of this solution in buret and titrate as before. If the solution is correct, *i.e.*, exactly normal, 20 cc. will exactly neutralize 20 cc. of the carbonate. If the average is less than 19.9 cc., redilute as before and confirm the titration. If the average is more than 20.1 cc., add several cc. of concentrated acid and repeat the preparation from the beginning.

**Notes.**—1. Titrations between 19.9 and 20.1 may be accepted as sufficiently accurate for routine laboratory work.

2. In filling a buret the tube below the stop cock must be perfectly filled and free from air bubbles.

**N/12 Sulphuric Acid Volumetric Solution.**—Fill a 100 cc. volumetric flask with normal sulphuric acid and transfer to a one-half gallon glass-stoppered bottle.

Fill the volumetric flask with 100 cc. of water and transfer to the bottle. Fill a 1 liter volumetric flask with water and add to the contents of the bottle. Mix thoroughly. This solution is N/12 sulphuric acid volumetric solution. A safe procedure is to check the finished product by titration against N/12 sodium carbonate volumetric solution.

**N/12 Sulphuric Acid Volumetric Solution from Concentrated Acid.**—To a liter of water in a ½ gallon glass stoppered bottle add 2.3 cc. of concentrated sulphuric acid. Mix thoroughly. Titrate the solution with N/12 sodium carbonate solution, using methyl orange indicator.

**N/12 Sodium Carbonate Volumetric Solution.**—Dry the anhydrous sodium carbonate in a drying oven at 105° C. for approximately 3 hours. Weigh on the analytical balance 4.417 grams and transfer to a liter volumetric flask. Dissolve in water and dilute to the graduation with the same. Mix thoroughly.

**Procedure.**—Titrate 20 cc. of the carbonate solution in a casserole with the acid solution in a buret, using methyl orange solution for the indicator. The end point is a faint pink. The amount of acid solution used should be between 19.9 and 20.1 cc.

If less than 19.9 cc. of acid solution are required for the titration, add water using Formula No. 1.

If more than 20.1 cc. of acid solution are required for the titration, add concentrated acid using Formula No. 2.

V = cc. of acid solution remaining after titration.

T = cc. of acid solution used in titrating.

D = difference in cc. between T and 20.

Formula No. 1  $\frac{V \times D}{T}$  = cc. of water to be added to acid solution.

Formula No. 2  $\frac{V \times D \times 0.0023}{T}$  = cc. of concentrated sulphuric acid to be added to the acid solution.

## HYDROCHLORIC ACID SOLUTIONS

**Standard Normal Hydrochloric Acid Solution.**—*Principle.*—The strength of a solution of hydrochloric acid, slightly stronger than normal, is determined by titration against a known amount of sodium carbonate. It is then diluted to exactly normal and the dilution confirmed by titration.

**Reagents.**—*Hydrochloric acid*, Sp. Gr. 1.18-1.19, 35 per cent.—*Methyl Orange.*—Dissolve 0.1 gram of methyl orange in water and dilute to 100 cc.

**Normal Sodium Carbonate Solution.**—Dry the sodium carbonate in an oven at 105° C. for 3 to 4 hours. Weigh on an analytical balance 5.300 grams; transfer quantitatively to a 100 cc. volumetric flask; dissolve in water and dilute to the graduation with water.

**Procedure.**—1. Dilute approximately 100 cc. of concentrated hydrochloric acid with water to 1 liter. Mix thoroughly.

2. Into a casserole pipet accurately 20 cc. of the normal carbonate solution. Add 2 drops methyl orange reagent. Titrate with the acid solution from a buret, using methyl orange solution for the indicator. The end point is a faint pink. The amount of acid solution used should be between 19.9 and 20.1 cc.



If less than 19.9 cc. of acid solution are required for the titration, add water using Formula No. 1.

If more than 20.1 cc. of acid solution are required for the titration, add concentrated acid using Formula No. 2.

3. Calculate the dilution as follows:

$V$  = cc. of acid solution remaining after titration.

$T$  = cc. of acid solution used in titrating.

$D$  = differences in cc. between  $T$  and 20.

Formula No. 1  $\frac{V \times D}{T}$  = cc. of water to be added to acid solution.

Formula No. 2  $\frac{V \times D \times 0.1}{T}$  = cc. of concentrated sulphuric acid to be added to the acid solution.

**Standard N/10 and N/100 Hydrochloric Acids.**—N/10 and N/100 hydrochloric acids may be prepared by accurately diluting the normal with distilled water. To make an N/10 solution, dilute 1 volume of normal to 10 volumes using distilled water.

To make an N/100 solution dilute 1 volume of normal to 100 volumes using distilled water.

## SODIUM HYDROXIDE SOLUTIONS

**Principle.**—Since sodium hydroxide contains carbonate and is not suitable for many hydroxide solutions, it is desirable to make a concentrated stock solution from which the carbonate will separate. This is diluted for use to the strength desired.

**Procedure.**—1. Weigh approximately 1 kilogram of sodium hydroxide and place it in a 2 liter beaker. Add 1 liter distilled water and allow to dissolve with occasional stirring. Care must be exercised because of the large amount of heat evolved. When at room temperature, transfer to a rubber-stoppered bottle. Allow this to stand for a few days, when the carbonate will settle to the bottom, leaving a clear supernatant liquid. This solution will contain 70 to 75 grams sodium hydroxide in each 100 cc.

2. Pipet accurately 5 cc. of the stock sodium hydroxide into a 1 liter volumetric flask. Dilute to mark and mix. Pipet 20 cc. of this diluted sodium hydroxide into a 150 cc. Erlenmeyer flask, add 1 drop of 0.5 per cent alcoholic phenolphthalein, heat to boiling, and titrate to the disappearance of the pink color with exact N/10 acid.

In case a normal solution of acid is available instead of the N/10 dilute 5 cc. of the stock sodium hydroxide to 100 cc. and proceed with the titration as above.

3. The calculation for either way is the same:

$t$  = cc. acid to titrate 20 cc. of dilute alkali

Gm. sodium hydroxide per 100 cc. stock sodium hydroxide solution =  $4t$

4. To make any per cent sodium hydroxide solution desired:

$$\frac{\text{per cent NaOH desired} \times 100}{\text{gram NaOH per 100 cc. stock solution}}$$

= cc. of the stock sodium hydroxide solution to 100 cc. with water.

For example:

$$10\% \text{ sodium hydroxide: } \frac{10 \times 100}{73} = 13.7 \text{ cc. stock sodium hydroxide diluted to 100 cc.}$$

$$4.5\% \text{ sodium hydroxide: } \frac{4.5 \times 100}{73} = 6.2 \text{ cc. stock sodium hydroxide diluted to 100 cc.}$$

**N/10 Sodium Hydroxide Solution.**—The sodium hydroxide should be as free as possible from carbonates; otherwise the solution will not have the same titrating value with all common indicators.

1. For each liter of N/10 sodium hydroxide desired, pipet 7.5 cc. of the clear concentrated solution described above and dilute to 1000 cc. with distilled water. For routine work it is convenient to make up 6 liters of solution.

2. To determine the exact strength of the solution pipet 25 cc. of the standard N/10 hydrochloric acid solution into a 100 cc. Erlenmeyer flask, add 2 drops of 0.5 per cent alcoholic solution of phenolphthalein, heat to boiling, and titrate. Repeat the titration until the results check within 0.1 cc.

3. To calculate the amount of distilled water which must be added to make it exactly N/10, calculate as follows:

V = cc. of hydroxide solution remaining after titration.

T = cc. of hydroxide solution used in titrating.

D = cc. difference between T and 25.

$$\frac{V \times D}{T} = \text{cc. of water to be added to the hydroxide solution.}$$

4. After addition of water check by titration to be certain that the solution is exactly N/10.

5. Keep in a rubber stoppered bottle.

6. Check the normality every few weeks.

**N/100 Sodium Hydroxide Solution.**—Dilute the N/10 standard 1:10. Titrate against N/100 hydrochloric acid solution using phenolphthalein as an indicator.

## PREPARATION OF TENTH NORMAL POTASSIUM PERMANGANATE AND SODIUM OXALATE

**Principle.**—Tenth normal sodium oxalate is prepared by accurately weighing pure sodium oxalate, dissolving and diluting to a definite volume. The potassium permanganate solution is prepared slightly stronger, titrated against the oxalate and finally diluted to the exact normality.

**Chemicals.**—*Potassium permanganate*,  $\text{KMnO}_4$ . *Sodium oxalate*,  $\text{Na}_2\text{C}_2\text{O}_4$ , (Sorenson special). *Sulphuric acid*,  $\text{H}_2\text{SO}_4$ .

**Procedure.**—Sodium oxalate, N/10:—dry about 10 gm. of the pure sodium oxalate in a drying oven at  $105^\circ \text{C}$ . for 3 to 4 hours. Weigh accurately 6.700 gms., dissolve in water and transfer quantitatively to a liter volumetric flask. Add 30 cc. of concentrated sulphuric acid. Cool and dilute to mark. Mix. This is exactly N/10 sodium oxalate.

Potassium permanganate, N/10:—dissolve about 3.5 gms. potassium permanganate

in about 1100 cc. water. Set aside for about 1 week before standardizing. Be careful that all is dissolved. Fill the buret with this solution.

Accurately pipet 20 cc. of the N/10 sodium oxalate solution into a casserole and warm to about 75 degrees. Titrate with the potassium permanganate until a faint pink color persists as the end point. Note the amount of permanganate used. Repeat and check within 0.1 cc.

**Calculation:**

$T$  = average titration in cc.

$W$  = cc. water to be added to the volumetric flask.

$W = 50 (20 - T)$ .

Place  $W$  cc. water in a liter volumetric flask. Add the permanganate solution to the mark. Mix thoroughly. This should be exactly N/10 permanganate. It is well to repeat the titration as above. The titration should be 20.00 cc.

*Notes.*—The permanganate after titration should be kept in a dark place. The solution may change after it has been freshly prepared but after standing the strength is generally constant. If it is not exactly N/10 an appropriate correction factor may be used.

**PREPARATION OF TENTH NORMAL SODIUM THIOSULPHATE  
AND POTASSIUM DICHROMATE SOLUTIONS**

**N/10 Sodium Thiosulphate Solution.**—Dissolve about 25 grams of sodium thiosulphate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ ) and 0.2 gram of sodium carbonate in a 1000 cc. of recently boiled and cooled distilled water.

Standardize the solution by titration against N/10 iodine, or against N/10 potassium dichromate by the following method:

Measure accurately 30 cc. of tenth-normal potassium dichromate into a glass-stoppered flask and dilute it with 50 cc. of distilled water. Add 2 gms. of potassium iodide and 5 cc. of hydrochloric acid, stopper and allow to stand for 10 minutes. Dilute with 100 cc. of distilled water and titrate the liberated iodine with the sodium thiosulphate solution. When the solution has assumed a yellowish-green color, add starch Test Solution and continue with the titration to the discharge of the blue color. Calculate the normality of the sodium thiosulphate solution and, if desired, adjust exactly to tenth-normal.

This solution should be frequently restandardized.

**N/10 Potassium Dichromate Solution.**—Dissolve 4.9035 gms. of reagent potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ ), which has been pulverized and dried to constant weight at 120° Centigrade, in sufficient distilled water to measure exactly 1000 cc. at standard temperature.

**Starch Test Solution.**—Triturate 1 gm. of arrowroot starch with 10 cc. of cold distilled water and pour slowly with constant stirring into 200 cc. of boiling distilled water. Boil the mixture until a thin, translucent fluid is obtained. Allow to settle and use only the clear, supernatant liquid. Longer boiling than necessary renders the test solution less sensitive. The test solution must be freshly prepared.

### PREPARATION OF STANDARD TENTH NORMAL IODINE SOLUTION

1. About 30 gm. of potassium iodide are dissolved in about 100 cc. of water.
2. In a weighing bottle, weigh out 13 gm. of crystalline iodine and transfer to a 1-liter volumetric flask. Wash out the weighing bottle with some of the iodide solution and pour it and the remainder of the iodide solution into the volumetric flask in such manner that any crystals of iodine adhering to the neck will be washed into the body of the flask.
3. The contents are now shaken until all the iodine has dissolved and then diluted to the mark with distilled water.
4. Standardize by titrating 20 cc. with  $\frac{N}{10}$  thiosulfate solution in the manner described for the standardization of the thiosulfate solution.
5. The factor for the iodine solution =

$$\frac{\text{cc. of 0.1N thiosulfate used}}{\text{cc. of iodine solution}}$$

### PREPARATION OF STANDARD TENTH NORMAL IODATE SOLUTION

Weigh out 3.566 gm. of potassium iodate, dissolve in distilled water in a 1-liter volumetric flask and dilute to the mark. This solution, made from pure iodate, does not need standardization. It keeps indefinitely in a glass stoppered bottle stored in the refrigerator.

### PREPARATION OF EXACTLY FIVE PER CENT PHENOL SOLUTION

**Tenth Normal Bromine Solution.**—Dissolve 3 gm. of potassium bromate and 50 gm. of potassium bromide in sufficient water to make 1000 cc. Ascertain its exact strength by titrating 25 cc. against  $\frac{N}{10}$  sodium thiosulphate solution after the addition of 5 cc. of 20 per cent potassium iodide and 5 cc. of concentrated hydrochloric acid. Calculate the factor for the bromine solution from

$$\frac{\text{cc. of 0.1N thiosulphate}}{25 \text{ cc. of bromine solution}} = \text{factor for the bromine solution.}$$

Preserve in an amber bottle in the refrigerator.

**Procedure.**—Dissolve about 55 gm. of phenol in a liter flask and dilute to the mark. Ascertain its exact strength as follows: Place 1.0 cc. of the solution in 250 cc. glass stoppered Erlenmeyer flask. Add from a buret 50 cc. of the  $\frac{N}{10}$  bromine solution and, from a cylinder, about 5 cc. of concentrated hydrochloric acid. Immediately insert the stopper. Shake the flask repeatedly during half an hour, keeping it away from direct sunlight. Let stand 15 minutes, remove the stopper sufficiently to allow the quick introduction, from a cylinder, of about 5 cc. of 20 per cent potassium iodide solution and at once stopper the flask. Shake thoroughly, remove the stopper and rinse

it and the neck of the flask with a little distilled water. Add  $\frac{N}{10}$  sodium thiosulphate from a buret, rapidly at first, then more slowly, as the iodine color begins to lighten. When the color becomes a pale yellow, add 1 cc. of starch solution and continue the addition of the thiosulfate drop by drop, with continual shaking until the complete discharge of the blue color. Note the number of cc. of thiosulphate solution used.

**Calculation.**—(cc. of bromine  $\times$  factor) — (cc. of thiosulfate  $\times$  factor) = cc. of  $\frac{N}{10}$  bromine utilized by 1 cc. of the phenol solution.

cc. of  $\frac{N}{10}$  bromine utilized  $\times 0.157 =$  gm. of phenol per 100 cc.

It will usually be found that the strength is slightly over 5 per cent. Therefore, according to the strength found, add water to the phenol solution sufficient to make it exactly 5 per cent. Check this strength by repeating the above titration twice. Take the average result. For use in the phenol coefficient test its strength should be 5 per cent  $\pm 0.05$ .

### PREPARATION OF "NORMAL" PHYSIOLOGICAL SALINE SOLUTION

Physiological saline solution, or normal saline, also known as 0.85 per cent sodium chloride solution for intravenous use, must be a sterile solution containing 0.85 per cent of sodium chloride, chemical pure, in distilled water. The solution must be free of any minute particles and foreign chemicals. For the preparation of the solution the water must be freshly distilled.

The principle of this method consists of the preparation and standardization, by means of a chemical titration, of a 13.6 per cent sodium chloride solution which is made once a week and is known as the stock saline solution. This solution is diluted each day with fresh distilled water, 1 liter of it to 15 liters of water, thereby obtaining a 0.85 per cent solution of sodium chloride known as the physiological saline solution. This saline solution is also titrated to determine its per cent of sodium chloride, thereby checking any error that might have been made in diluting the stock saline solution. Both stock and physiological solutions are mixed in their preparation by drawing air through them, using a filter pump, water jet form, marketed by A. H. Thomas Co. The air is washed by drawing it through soda lime, a weak sulphuric acid solution and finally distilled water. An empty flask is placed between the suction pump and the solution being mixed, as a precaution against the filter pump "back firing."

Stock and physiological solutions are filtered by means of sintered glass filters, no paper filters being used at any time. A sintered glass filter consists of a disk of porous glass sealed in the mouth of a funnel. The filter is immersed in the solution to be filtered, the end of the neck of the filter is connected by means of rubber tubing to a perforated rubber stopper in the mouth of an empty carboy in which a vacuum is created by means of the filter pump, the filtrate being drawn through the neck of the filter, the rubber tubing and into the empty carboy.

The physiological saline solution having been standardized and filtered, is bottled in Pyrex Erlenmeyer flasks. Each flask is labeled with permanently baked-in letters "Normal Saline 0.85 per cent." The flasks are stoppered with a paper cap or hood, the skirt or side of the cap completely covering the neck of the flask. The cap is held

firmly around the neck of the flask by means of 2 wire loops that are tightened by twisting, using a tool such as is used in tightening the wire on a champagne bottle. The operation of tightening the wires requires but a few seconds of time. Each cap is stamped on the top with the date that the solution was made.

The flasks are then placed in large wire baskets capable of holding eight 2-liter flasks, each flask being in a separate compartment.

Each flask of solution is then sterilized within a few hours after its preparation.

### PREPARATION OF STOCK SALINE SOLUTION

A 13.6 per cent solution of chemical pure sodium chloride and freshly distilled water is prepared once a week. Ten liters are prepared in a 5 gallon Pyrex carboy. The solution is mixed by drawing air through it as described above. Its exact sodium chloride content is determined by means of a chemical titration which will check any error in weighing or dilution.

Several solutions are required for the titration. These are called stock solutions, to differentiate them from a group of similar solutions but of a weaker concentration used in titrating the standard saline solution.

**Stock Silver Nitrate Solution.**—Weigh on an analytical balance 39.529 grams of silver nitrate, dissolve in distilled water and dilute to a volume of 100 cc. Keep in a glass-stoppered brown bottle.

**Stock Ammonium Thiocyanate Solution.**—180 grams of ammonium thiocyanate are dissolved in distilled water and diluted to a volume of 1 liter. This solution must be standardized against the stock silver nitrate solution, so that 1 volume of it will be equivalent to 1 volume of the silver solution. This is accomplished by pipeting 15 cc. of stock silver nitrate solution into a 250 cc. Erlenmeyer flask to which is added 15 cc. of concentrated C.P. nitric acid, 0.3 gram of powdered ferric ammonium sulphate and approximately 30 cc. of distilled water. A 25 cc. is buret filled with the stock ammonium thiocyanate solution which is slowly titrated into the Erlenmeyer flask until a salmon pink end point is obtained that will persist for 15 seconds. If the buret reading is 15 cc., the stock ammonium thiocyanate is correct. If less than 15 cc. were used the thiocyanate solution is too concentrated and must be diluted using the formula:

$$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (15 - \text{Titre}) = \text{cc. of distilled water to be added.}$$

If the reading of the buret was greater than 15 cc. then the stock thiocyanate solution is weak in concentration and ammonium thiocyanate must be added using the formula:

$$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (\text{Titre} - 15) \times 0.18 = \text{grams of ammonium thiocyanate to be added.}$$

All the reagents are now ready to titrate the stock saline solution.

**Titration of Stock Saline Solution.**—Into a 250 cc. Erlenmeyer flask pipette 10 cc. of stock saline solution, 15 cc. of stock silver nitrate solution, 15 cc. of concentrated C.P. nitric acid. Add 0.3 gram of powdered ferric ammonium sulphate. Allow

the flask to stand 5 minutes in a dark place, then titrate with the stock ammonium thiocyanate solution. If the stock saline solution is of a correct concentration then 10 cc. of it will combine with 10 cc. of the stock silver nitrate solution, therefore requiring 5 cc. of the stock ammonium thiocyanate solution to bring the titration to the proper end point. If less than 5 cc. of the thiocyanate solution were used the stock saline solution is of too strong a concentration and is diluted, using the formula:

$$\frac{\text{Volume Stock Saline Sol.}}{10} \times (5 - \text{Titre}) = \text{cc. of distilled water to be added to the stock saline solution.}$$

If more than 5 cc. of stock ammonium thiocyanate solution was required for the titration then the stock saline solution is weak in concentration and must be strengthened with sodium chloride using the formula:

$$\frac{\text{Volume Stock Saline Sol.}}{10} \times (\text{Titre} - 5) \times 0.136 = \text{grams of sodium chloride to be added to the stock saline solution.}$$

After the stock saline solution is found to be of the correct concentration, it is filtered through the sintered glass filters as described above and is then ready for dilution to make standard saline solution.

**Preparation of Standard Solutions for Titrating 0.85 Per Cent Saline Solution.**—Using a liter volumetric flask transfer 1 liter of stock saline solution to a 5-gallon Pyrex glass carboy, then add 15 liters of freshly distilled water, and mix the solution by drawing air through it for about 20 minutes. This solution is physiological saline solution and should contain 0.85 per cent of sodium chloride. To check any error that might have been made it is titrated with reagents similar to the ones used in titrating the stock saline solution, except that these reagents are weaker in concentration and are called "standard solutions" to differentiate them from the stock solutions mentioned above.

**Standard Silver Nitrate Solution.**—Weigh on an analytical balance 2.47 grams of silver nitrate C.P., dissolve in distilled water and dilute to a volume of 100 cc. Keep in a glass-stoppered brown bottle. 1 cc. of this solution is equivalent to 1 cc. of 0.85 per cent sodium chloride solution.

**Standard Ammonium Thiocyanate Solution.**—Dissolve 11.25 grams of ammonium thiocyanate C.P. in distilled water and dilute to a volume of 1 liter. This solution must be standardized against the standard silver nitrate solution in the same manner that the stock ammonium thiocyanate solution was standardized against the stock silver nitrate solution, *i.e.*, 15 cc. of standard silver nitrate are pipeted into a 250 cc. Erlenmeyer flask to which is added 15 cc. of concentrated nitric acid C.P. and 0.3 gram of powdered ferric ammonium sulphate. Approximately 15 cc. of distilled water are added. The flask is mixed and kept in a dark place for 5 minutes. Fill a 25 cc. buret with the standard ammonium thiocyanate solution and titrate into the flask to a salmon pink end point. If the buret reading is 15 cc. the thiocyanate solution is correct and 1 cc. of standard ammonium thiocyanate solution is equivalent to 1 cc. of standard silver nitrate solution. If less than 15 cc. of the thiocyanate solution was used it is too strong in concentration and must be diluted with distilled water using the formula:

$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (15 - \text{Titre}) = \text{cc. distilled water to be added to standard ammonium thiocyanate solution.}$

If more than 15 cc. of thiocyanate solution were used, it is weak in concentration and ammonium thiocyanate must be added using the formula:

$\frac{\text{Volume Ammon. Thio.}}{\text{Titre}} \times (\text{Titre} - 15) \times 0.01125 = \text{grams ammonium thiocyanate to be added to the standard ammonium thiocyanate solution.}$

These standard reagents are now ready for use.

**Titration of Physiological Saline Solution.**—Pipet 20 cc. of physiological saline solution into a 250 cc. Erlenmeyer flask, add 25 cc. of standard silver nitrate solution, 15 cc. of concentrated nitric acid C.P. and 0.3 gram of powdered ferric ammonium sulphate. Mix and allow to stand in a dark place for 5 minutes, then titrate it with standard ammonium thiocyanate solution to a salmon pink end point. If the reading of the buret is 5 cc. the saline is of the correct concentration. If the reading is less than 5 cc., the saline solution is strong in concentration and must be diluted with distilled water using the formula:

$\frac{\text{Total Volume of Saline}}{20} \times (5 - \text{Titre}) \text{ cc. of distilled water to be added to the physiological saline solution.}$

If more than 5 cc. of standard ammonium thiocyanate solution were used, the physiological saline solution is weak in concentration of sodium chloride and more stock saline solution must be added using the formula:

$\frac{\text{Total Volume of Saline}}{20} \times \frac{(\text{Titre} - 5)}{15} = \text{cc. of stock saline solution to be added to the physiological saline solution.}$

If it was necessary to make any adjustment to the physiological saline solution it must be mixed by drawing air through it and again titrating to make certain that it is of the correct concentration.

The physiological saline solution is then filtered through the sintered glass filters and is ready for bottling.

**Dispensing.**—The physiological saline solution is dispensed by syphoning it into a volumetric flask of the desired volume and then pouring the contents into a permanently labeled flask, which has been thoroughly washed with hot water and soap and rinsed with tap water and finally, distilled water. The flasks are capped immediately in order that no dust particles may enter. The caps are held in place by means of 2 wire loops, 1 at the upper and 1 at the lower part of the skirt of the cap. The loops are tightened by means of a hook on the end of a twisted rod, on which a nut, in the form of a handle, is screwed. The operator holds the handle and pulls, thereby turning the rod and likewise twisting the wire loop. Each flask must be examined before it leaves the laboratory for the sterilization room where it is autoclaved a short time after its manufacture.

The paper caps can be stamped with the date of manufacture.

This method of manufacturing physiological saline solution is efficient, scientific and inexpensive.



The 13.6 per cent stock saline solution can be safely kept for a week or even longer as no bacteria will grow in it due to its high concentration of sodium chloride.

### MODIFIED DAKIN'S SOLUTION

An aqueous solution of chlorine compounds of sodium containing, in each 100 cc., not less than 0.45 gm. and not more than 0.50 gm. of NaOCl, equivalent to not less than 0.43 gm. and not more than 0.48 gm. of available Cl.

Diluted solution of sodium hypochlorite may be prepared as follows:

Solution of sodium hypochlorite..... 1000 cc.

Sodium bicarbonate.

Distilled water, of each, a sufficient quantity.

Dilute the solution of sodium hypochlorite with 5000 cc. of distilled water and add 40 cc. of a 5 per cent solution of sodium bicarbonate in cold distilled water and mix well. Remove about 20 cc. of the mixture, add to it about 0.02 gm. of powdered phenolphthalein, and shake it gently for 2 minutes. If a red color appears, add more of the sodium bicarbonate solution, and test with powdered phenolphthalein as just described, repeating the procedure as often as necessary until no red color is produced. Assay the liquid and dilute it with sufficient distilled water to make the final solution contain, in each 100 cc., 0.48 gm. of NaOCl.

**Assay.**—Measure accurately 25 cc. of the solution and dilute it with 25 cc. of distilled water. Add 1 gm. of potassium iodide and 10 cc. of acetic acid, and titrate the liberated iodine with tenth-normal sodium thiosulphate, using starch T.S. as the indicator. Each cc. of tenth-normal sodium thiosulphate is equivalent to 0.003723 gm. of NaOCl.

**Note.**—Preserve diluted solution of sodium hypochlorite in well-stoppered bottles, in a cool place and protected from light.

# METHODS FOR CHEMICAL EXAMINATIONS OF THE BLOOD

## METHOD FOR PREPARATION OF GLASSWARE

1. Glassware should be washed as soon after use as possible. Wash with tap water, using a solution of soap made by dissolving a soap powder or chips in tap water. Rinse thoroughly with tap water and finally with distilled water and allow to dry.

2. A tall crock or cylinder full of tap water with a layer of cotton in the bottom should be kept near to receive soiled pipets immediately after using. To wash pipets hold them in the flowing tap water, or better, use a water suction pump attached to the faucet. Place the one end of the pipet in the rubber tube connected with the pump and the other end in a container of tap water and allow the pump to suck the water from the container through the pipet for about half a minute. Remove the pipet from the rubber tube and allow a little distilled water to run through the pipet, then put aside to dry.

3. When glass retains a cloudiness that cannot be removed by means of washing with soap, use potassium dichromate sulphuric acid cleaning solution, made as follows:

Technical potassium dichromate..... 15 gm.

Technical sulphuric acid ..... 500 cc.

Fill beakers and flasks with cleaning solution and place pipets in a tall cylinder full of solution and allow to remain 24 hours.

4. The cleaning fluid can be used repeatedly and when it appears to lose its strength add more potassium dichromate and sulphuric acid.

## METHODS FOR COLLECTION OF BLOOD

1. Blood is usually taken from a vein at the elbow with a sterile syringe, the technic being described and illustrated on pages 44 to 47. Blood may also be taken from a finger, if a micromethod of analysis is to be conducted as in blood sugar determinations. Umbilical cord blood may be secured at birth or specimens obtained from infants and young children by puncture of the external jugular veins or superior longitudinal sinus, the methods being described and illustrated on pages 47 to 48.

2. After standardization of diet and exercise, a pneumatic tourniquet is applied to the arm at a pressure sufficient to obstruct the venous, but not the arterial circulation. Blood is withdrawn with a syringe and needle and transferred to a test tube.

3. If the method of analysis requires serum a rubber stopper is inserted into the test tube and the blood is allowed to clot. If whole blood or plasma is required for the analysis an anticoagulant must be present, a rubber stopper is inserted and the test tube inverted several times in order that the anticoagulant may come in contact with all the blood.

4. Sodium oxalate is the most generally used anticoagulant; an excess should be avoided. It is preferred to potassium oxalate because (a) the lower solubility of the sodium salt prevents excessive amounts dissolving in the blood which would interfere with the preparation of the protein-free filtrate, and (b) the presence of potassium salts is an important cause of the clouding that sometimes occurs during the color development in the determination of uric acid.

5. **Preparation of Oxalated Tubes.**—Pipet 0.5 cc. of a hot saturated sodium oxalate solution into test tubes. At low heat and with constant rotation evaporate the solution, leaving the dry sodium oxalate deposited in a finely divided state about the sides of the tubes. When cool insert a rubber stopper.

6. Blood sugar is rapidly destroyed (glycolysis) on standing and specimens intended for sugar determination or sugar and other determinations should have sodium

fluoride (C.P. powder) added as a preservative and anti-coagulant in the proportion of 60 milligrams per 10 cc. of blood if the determination cannot be made almost immediately. As sodium fluoride is not very soluble, it is necessary to mix thoroughly to prevent clotting. If the blood is on the point of coagulating, it is well to add oxalate also. *Fluoride should not be added to specimens intended for urea determination as results will be too low.* The addition of thymol to the fluoride is recommended for preventing or greatly inhibiting glycolysis for several days. Chlorobenzol may be used as a preservative for specimens to be kept up to 5 days for sugar determinations. When possible, blood samples are examined immediately after withdrawal. However, blood may be preserved in paraffin-coated tubes at a temperature of 0° C. to 5° C. for several days with practically no change in the usually sought constituents except carbon dioxide and hydrogen ion concentration.

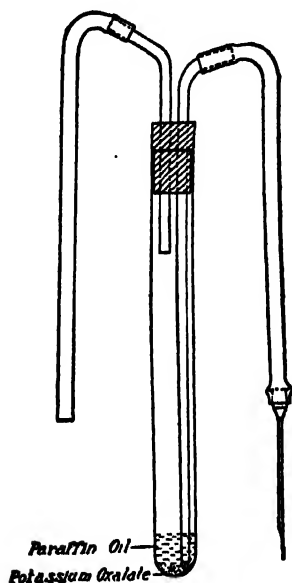


FIG. 317.—TUBE USED IN COLLECTING BLOOD FOR DETERMINATION OF PH OR CARBON DIOXIDE

7. A blood sample for the determination of hydrogen ion concentration is drawn in a special manner, in a centrifuge tube or heavy-walled test tube, under paraffin oil to avoid contact with air, and without stasis in the vein (Fig. 317).

8. *In the following pages whenever directed to "dilute to the mark," distilled water is to be used unless some other solution is especially mentioned. The word "water" always implies distilled water.* All solutions must be thoroughly mixed after diluting to the final volume, unless instructed to the contrary.

9. The chemicals used are all of the grade of C.P. Analyzed Chemicals, unless otherwise mentioned.

10. Table 51 shows the normal values and also indicates the amount of blood, serum or plasma ordinarily required for the various determinations.

## METHOD FOR PREPARATION OF PROTEIN-FREE FILTRATE

**Haden's Modification of the Folin-Wu Method.**—In this method (*Jour. Biol. Chem.*, 38: 81, 1919; *ibid.*, 56: 469, 1923) the proteins of blood are completely removed by filtration following precipitation with tungstic acid, which is formed by the interaction of sodium tungstate and sulphuric acid.

**Reagents.**—1. *Sulphuric acid N/12*: Prepared as described on page 782.

2. *Sodium tungstate solution*: dissolve 10 grams of sodium tungstate (special, according to Folin) in water and dilute to 100 cc.

TABLE 51

Constituent	Normal Value (mg. per 100 cc. for adults unless otherwise stated)	Amount and Preparation of Blood *
Amino acid nitrogen	4-8	5 cc., oxalated
Amylase	40-110 mg. (av. 60) of glucose from starch by 100 cc. serum	5 cc., plain
Bilirubin (Van den Bergh)	direct: negative indirect: 0.1-0.25	5 cc., plain
Calcium	7-11	5 cc., plain
CO <sub>2</sub> capacity (plasma)	adults: 53-70 vol. % infants: 48-63 vol. %	5 cc., oxalated
Cholesterol	total: 150-250 esters: 60-75% of total	5 cc., plain
Chlorides (as sodium chloride)	serum: 570-620	5 cc., plain
Creatinine	1-2	5 cc., oxalated
Fibrinogen	200-400	6 cc., oxalated
Glucose	80-110 85-120 (finger tip)	2 cc., oxalated 0.1 cc., (micro)
Icteric index	4-6 units	5 cc., plain
Iron	organic: 50-52 in men (lower in women)	5 cc., oxalated
Lipase	0.5-1.0 units	5 cc., plain
Nonprotein nitrogen	25-35	2 cc., oxalated
Oxygen capacity	men: 18-7-22.7 vol. % women: 17-21 vol. %	5 cc., oxalated
Oxygen unsaturation	2.5-9.0 vol. %	12 cc., oxalated
Phosphatase	Bodansky: (acid: 0.0-1.2 units) (alk.: 2.0-9.0 units) King and Armstrong: (acid: 0.0-50 units) (alk.: 0.0-13.0 units)	5 cc., plain
Phosphorus	adults: 3-4 children: 5-5.5	5 cc., plain
Potassium	16-22	5 cc., plain
Sodium	310-340	5 cc., plain
Sulfur (inorganic)	0.9-1.6 (2.7-4.8 as sulfate)	5 cc., plain
Total protein albumin globulin A-G ratio	6.0-7.5 gm. per 100 cc. 4.0-5.0 " " " " 2.0-2.5 " " " " 1.5 to 2.5:1	5 cc., plain
Urea nitrogen	10-15	5 cc., oxalated
Uric acid	2-4	5 cc., oxalated

\* Provides excess for recheck and duplicate determination; about one-half amounts sufficient for single determinations; oxalated for whole blood or plasma; plain for serum.

3. The filter paper should be ammonia-free; diameters 9 to 12.5 centimeters. Whatman No. 2 is recommended.

**Procedure.**—1. Transfer 8 volumes of N/12 sulphuric acid to a flask with a capacity of about 125 cc.

2. Add 1 volume of oxalated whole blood. Ostwald-Folin pipets (Fig. 318) should be used, and rinsed with the acid blood mixture several times. Allow to stand until the blood is laked as shown by the formation of brown acid hematin.

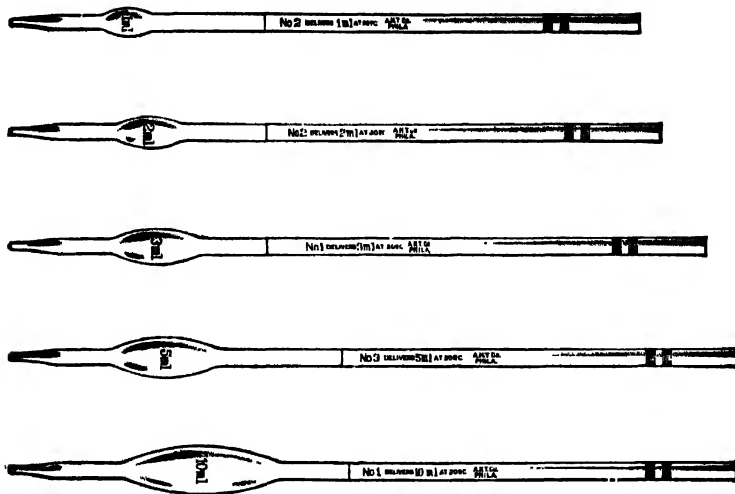


FIG. 318.—OSTWALD-FOLIN PIPETS

3. Add 1 volume of the sodium tungstate solution. Stopper the flask and shake thoroughly. The coagulum should be dark brown with little or no frothing.

4. Filter the mixture. The filtrate should be perfectly clear. Instead of filtering the mixture may be centrifuged.

5. If the filtrate is not to be used within a short time it should be placed in the refrigerator. If it is to be kept longer than 2 days, a few drops of toluene should be added to prevent bacterial decomposition. Filtrates from oxalated blood may be kept overnight in the refrigerator without appreciable loss of sugar or uric acid; standing several days has little effect on the non-protein nitrogen, creatinine or creatine values.

**Notes.**—1. If there is much foaming and the coagulum assumes a brownish-pink instead of a dark brown color, it is usually because too much oxalate is present. In such a case the sample can generally be saved by adding 10 per cent sulphuric acid, 1 drop at a time, shaking vigorously after each drop, and continuing until there is practically no foaming and until the dark brown color has appeared.

2. If the filtrate is not clear, the precipitate and the filtrate should be returned to the flask and 10 per cent sulphuric acid added as above to complete the protein precipitation.

3. The filtrate should be nearly neutral when the reagents are properly adjusted. With Congo red the filtrate should give a negative test and with blue litmus a positive test. Excess acidity will result in precipitation of uric acid.

**KARR METHOD FOR DETERMINATION OF UREA NITROGEN**

**Principle.**—In this method (*Jour. Lab. and Clin. Med.*, 9: 3, 1924) protein-free blood filtrate is incubated with urease and acetate buffer solution. The resultant solution is nesslerized and compared colorimetrically with a similarly treated standard urea solution.

**Apparatus.**—Two test tubes, each graduated at 22.5 and 25 cc.

**Reagents.**—1. *Urease.*—Place about 3 grams of permutit in a 500 cc. flask. Add 50 cc. of 2 per cent acetic acid. Shake. Allow the permutit to settle, then discard the supernatant fluid. Wash the permutit twice with 50 cc. of water, discarding each time. To the washed permutit add 15 grams of jack bean meal. Add a mixture of 16 cc. of alcohol and 84 cc. of water. Shake gently but continuously for 15 minutes. Allow to stand overnight in a refrigerator. When the supernatant fluid is clear, transfer it to small flasks, keeping them tightly stoppered in a refrigerator.

2. *Buffer.*—Dissolve 20 gm. of sodium acetate in water and add 2.2 cc. of 10 per cent acetic acid. Dilute to 100 cc. and mix.

3. *Urea Nitrogen Standard Stock Solution.*—Dissolve 0.1286 gm. of urea in water and dilute to 200 cc. (5 cc. contains 1.5 mgms. urea nitrogen).

4. *Urea Nitrogen Standard Working Solution.*—Place 5 cc. of stock urea solution in a 100 cc. volumetric flask and dilute to mark (5 cc. contains 0.075 mg. urea nitrogen).

5. *Nessler Solution* (Koch-McMeekin).—Dissolve 30 gm. of potassium iodide in 20 cc. of distilled water and add 22.5 gm. of iodine to the solution. Shake until dissolved and then add 30 gm. of pure metallic mercury. Shake the mixture well, keeping the solution cool by holding under running tap water from time to time, until the supernatant liquid has lost the yellow color of iodine. Pour off from the undissolved mercury and test for the presence of excess iodine by adding a few drops to a little starch solution (page 786) in a test tube. If no blue color is obtained add iodine solution similar to that above, drop by drop, until there is a slight excess of free iodine as indicated by the test with starch solution. Dilute to 200 cc., mix, and pour into 975 cc. of accurately prepared 10 per cent sodium hydroxide solution (page 784). Mix well and allow any precipitate to settle out. Use the clear supernatant fluid. Avoid stirring up the sediment when removing from the storage bottle.

6. *Gum Ghatti Solution.*—Place about 20 gm. of gum ghatti (tears) in a bag made from a double layer of gauze and suspend the bag in a cylinder containing 1000 cc. of water in such a position that the upper surface of the water just covers the contents of the bag. Allow to stand overnight. Remove the bag after gentle squeezing and discard. Filter the solution through cotton into a tall narrow bottle to permit any further settling of sediment. Use only the clear upper layer. The solution keeps well.

**Procedure.**—Into an ordinary test tube marked *S*, pipet 5 cc. of the standard solution of urea; into another tube marked *B*, pipet 5 cc. of the protein-free blood filtrate. Into each add 5 drops of the urease solution and 0.5 cc. of the buffer solution. Place tubes in water bath at 50° C. for 10 minutes. At the end of this time transfer, quantitatively, the solutions in each tube to the graduated tubes (marked *S* and *B*). Wash out the tube twice with about 5 cc. of water into the graduated tube. Add 2 drops of gum ghatti solution and dilute to the lower mark with water. Add the Nessler solution to the 25 cc. mark. Mix by inverting. Compare in colorimeter after 10 minutes.

**Calculation:**

$x$  = milligrams urea nitrogen in 100 cc. of blood

$R$  = reading of the blood filtrate (contents of tube  $B$ )

$S$  = reading of the standard (contents of tube  $S$ )

$$x = \frac{15S}{R} \text{ or } x = S \text{ if } R \text{ is set at } 15 \text{ mm.}$$

**Notes.**—1. Visual colorimetry is difficult and somewhat uncertain with this type of color. More accurate color measurements can be made in the photoelectric colorimeter. If a Klett-Simmerson type is available, use light filter No. 54 and determine a blank reading against a distilled water zero. The blank should contain all the ingredients of the unknown except the filtrate which should be replaced by water. This blank is small and constant and need only be determined occasionally. The blank reading is subtracted from the reading of both unknown and standard. The calculation then is:

mg. of urea per 100 cc. of blood =  $\frac{15}{\text{reading of standard}} \times \text{reading of unknown}$ . The value of the fraction is a constant for any one set of reagents and, when once determined, is used as a factor which is multiplied by the reading of the unknown. The use of such factor, if determined by averaging the readings of several standards, further simplifies the method since standards need only be run occasionally thereafter as a check on the constancy of the factor.

2. The normal range is about 10 to 15 mgs. urea nitrogen per 100 cc. of blood.

3. The tubes in which the filtrate and standard are incubated must be kept clean and never used to contain the Nessler solution.

4. With bloods known or thought to have a high urea nitrogen content, use less filtrate, adding sufficient water to make 5 cc., and make corresponding calculation. For a large number of determinations at one time, an artificial standard may be used. The amount of urea nitrogen in the blood is affected by diet. Urea nitrogen is increased above normal in kidney insufficiency. Retention may be said to exist when the concentration reaches 20 milligrams in 100 cc. of blood.

### **KELLER MICROMETHOD FOR DETERMINATION OF UREA NITROGEN**

**Principle.**—In this method (*Jour. Lab. and Clin. Med.*, 17: 1146, 1932) protein-free blood filtrate is incubated with urease and a buffer solution. The resultant solution is nesslerized and compared colorimetrically with a similarly treated standard urea solution.

**Materials.**—Two small test tubes each graduated at 9 and 10 cc.

**Reagents.**—Same as for Karr's Method. **Tungstic Acid Solution.**—Transfer 20 cc. of 10 per cent sodium tungstate to a liter volumetric flask. Add about 700 cc. of water. Add, with shaking, 160 cc. of N/12 sulphuric acid and dilute with water to 1 liter.

**Procedure.**—1. Place 9.8 cc. of tungstic acid solution in a 15 cc. centrifuge tube. Prick finger with a lancet so that the blood flows freely. Using a 0.2 cc. serological pipet, collect 0.2 cc. of blood. Introduce into the centrifuge tube and rinse pipet. Stopper and mix. Then centrifuge at a high velocity for a period of 5 minutes.

2. Into a small test tube "B" pipet 4 cc. of the supernatant fluid. Into another

small test tube "S" pipet 1 cc. of urea nitrogen standard solution. To each tube add 3 drops of urease and 3 drops of buffer solution.

3. Digest for 10 minutes at 50° C.

4. Transfer contents to respective graduated tubes, rinsing with water and diluting to 9 cc. mark. Add Nessler's solution to 10 cc. mark.

5. Compare colorimetrically after 10 minutes.

**Calculation.**—Set the unknown sample at 15 millimeters, when 1.25 times the reading of the standard gives milligram of urea nitrogen per 100 cc. of blood.

## ORMSBY METHOD FOR DETERMINATION OF UREA NITROGEN

**Principle.**—In this method (*Jour. Biol. Chem.*, 146: 595, 1942) when urea is heated with biacetyl monoxime in acid solution, a yellow color develops. This color, deepened by oxidation with persulfate, is compared in the colorimeter with a standard urea solution similarly treated.

**Reagents.**—*Hydrochloric acid*, concentrated.

*Biacetyl monoxime* (Eastman). A 3 per cent aqueous solution keeps indefinitely in the refrigerator.

*Potassium persulfate solution*, 1 per cent. This solution should be kept in the refrigerator and keeps about 2 weeks.

*Standard Urea Nitrogen Solution.*—This is the same as the diluted standard used in Karr's method (page 797) (3 cc. = 0.045 mg. of urea N.)

**Procedure.**—1. Place 3 cc. of 1:10 tungstic acid filtrate in a test tube.

2. Place 3 cc. of urea nitrogen standard in a second test tube.

3. Place 3 cc. of water in a third test tube (blank).

4. To each tube add exactly 5 cc. of concentrated hydrochloric acid and 0.5 cc. of the biacetyl monoxime solution.

5. Mix the contents by rotation, cover each tube with a marble and place the tubes in vigorously boiling water for 10 minutes.

6. Remove the tubes simultaneously and cool for 2 minutes in running water.

7. Add slowly 0.25 cc. of the persulfate solution so that a separate layer is formed, stopper and mix simultaneously by inverting a few times.

8. Read in the Klett-Summerson photoelectric colorimeter using filter No. 42, at intervals of 5, 15 and 25 minutes, using a distilled water zero. The amount of color developed varies somewhat with the concentration of urea; it is necessary to use the maximum reading.

**Calculations.**—Subtract the reading of the blank from the reading of the standard and unknown; then  $\frac{15}{\text{reading of standard}}$  (= factor)  $\times$  reading of unknown = mg. urea nitrogen per 100 cc. of blood.

If a visual colorimeter is used, make readings after 15 minutes. Omit the blank tube. If the standard is set at 20:  $\frac{300}{\text{reading of unknown}}$  = mg. per cent urea N. If the unknown is set at 15: Reading of standard = mg. per cent urea N.

**Notes.**—1. The above method avoids the difficulties inherent in a nesslerization procedure and is not affected by ammonia or other normal blood constituents.

2. The method, in addition to being very simple, (requiring but two simply pre-



pared reagents) and rapid (no incubation period necessary), probably also exceeds the nesslerization methods in accuracy.

3. The method as outlined will determine values up to about 40 mg. per 100 cc. of urea nitrogen. If more than this is found, repeat the test using 1 cc. of the Folin-Wu filtrate plus 2 cc. of water. The above factor must, of course, be multiplied by 3.

4. The factor is constant for constant conditions and the same reagents. Standards and blanks need not be run with the unknowns after the constants have been established.

### VAN SLYKE METHOD FOR DETERMINATION OF UREA NITROGEN

**Principle.**—In this method (*Jour. Biol. Chem.*, 83: 449, 1929) alkaline hypobromite is caused to react with the urea in the tungstic acid filtrate in the reaction chamber of the Van Slyke-Neill closed type manometric apparatus and the pressure of liberated nitrogen measured at definite volume.

**Materials.**—Van Slyke-Neill closed type manometric apparatus (Fig. 319).

Two Ostwald-Van Slyke pipets with stop cocks, one graduated at 1 cc., the other at 5 cc. Both should be rubber tipped (Fig. 320).

**Reagents.**—1. *Forty per cent sodium hydroxide solution.*

2. *Bromine solution:* Dissolve 60 gms. potassium bromide in 100 cc. water. Add 2.5 gms. liquid bromine. The resultant yellow solution keeps many months in a ground glass-stoppered bottle. Sufficient alkaline hypobromite is prepared for the day's use by mixing these two solutions in the proportions of 1.25 cc. of bromine solution to 0.75 cc. of sodium hydroxide.

**Procedure.**—1. The reaction chamber is filled with mercury by raising the leveling bulb to the upper position, opening the stop

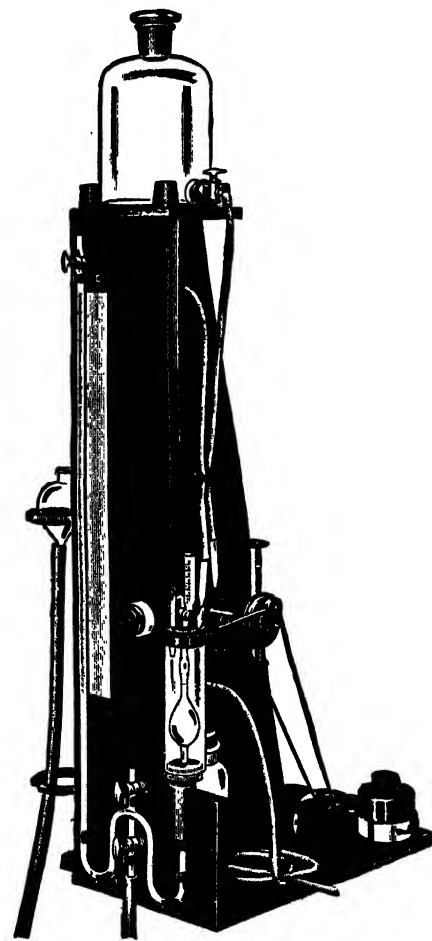


FIG. 319.—VAN SLYKE-NEILL CLOSED TYPE MANOMETRIC APPARATUS

cock above the reaction chamber and manipulating the stop cock leading from the leveling bulb to the reaction chamber and a small amount of mercury is allowed to run over into the cup above the chamber.

2. Using the 5 cc. rubber-tipped pipet, the tungstic acid filtrate is drawn up to the upper graduation.

3. Place the rubber-tipped end into the cup and press it firmly against the stop cock at the top of reaction chamber; open this stop cock and the one on the pipet and by lowering the mercury in the reaction chamber allow the filtrate to run into the

chamber until 5 cc. has been admitted. The flow of filtrate may be readily controlled by the stop cock with which the mercury level is controlled. Closing this stop cock stops the flow of filtrate instantly.



FIG. 320.—OSTWALD-VAN SLYKE PIPET

4. Close the stop cock on the 5 cc. pipet and remove it from the cup.
  5. Close the stop cock at the top of the reaction chamber and by lowering the leveling bulb manipulate the lower stop cock to bring the mercury level to the 50 cc. mark. Close the lower stop cock.
  6. Shake for 1 minute to remove adsorbed air.
  7. Allow the mercury to flow back into the reaction chamber slowly as far as it will go, then by slowly turning the upper stop cock of the reaction chamber, permit the air bubble to escape, being careful that none of the filtrate is lost.
  8. Using the 1 cc. rubber-tipped pipet, draw up the alkaline hypobromite to the upper mark and allow it to flow into the reaction chamber in the same manner that the filtrate was introduced.
  9. After closing the pipet stop cock remove it and allow a little mercury from the cup to drain into the reaction chamber, thus sealing the hole of the stop cock with mercury.
  10. Draw the mercury down to the 50 cc. mark and shake for 2 minutes.
  11. Allow the mercury to run back into the reaction chamber until the level of the aqueous liquid is at the 0.5 cc. mark.
  12. Read the level of the mercury in the manometer tube and the temperature of the water jacket, and then allow it to refill the tube.
  13. Expel the gas from the reaction chamber by opening the stop cock at the top of the reaction chamber.
  14. Close this stop cock and bring the level of the aqueous liquid again to the 0.5 cc. level. Read the manometer. This is the zero reading and need only be made occasionally, it being constant for any one arrangement of the apparatus.
- If high urea figures are expected (this can be ascertained if the mercury in the manometer begins to rise rapidly as the liberated nitrogen is compressed in the reaction chamber), then the manometric reading is taken when the liquid level in the chamber reaches the 2 cc. mark. From this reading is then subtracted the manometer reading obtained after the nitrogen is expelled and the level of the liquid returned to the 2 cc. mark.

**Calculation.**—The difference between the two readings is multiplied by the appropriate factor taken from the table to give mgm. of urea or urea N. per 100 cc. of blood.

**Notes.**—1. The measurements, being based on direct observation of substance obtained, are independent of standard solutions. Thus it is peculiarly adapted to intermittent determinations without the necessity of checking possible deterioration of standards.

2. The simple reagents keep well and with practice a determination may be completed in 4 to 5 minutes.

3. The reaction chamber need not be cleaned between successive determinations. An occasional cleaning with dilute lactic acid (by pouring it into the cup and allowing

it to drain into the reaction chamber) serves to keep the mercury free of the mercuric oxide which slowly forms. This cleaning, followed by rinsing with distilled water should be done daily if many determinations are made. Otherwise, a daily rinsing with distilled water is sufficient.

4. The usual source of error is leakage at the reaction chamber stop cock. This can be kept airtight by the use of special lubricant (lubriseal) supplied for vacuum stop cocks.

**TABLE 52.—FACTORS BY WHICH MANOMETER READINGS ARE MULTIPLIED TO GIVE UREA AND UREA N. CONTENT OF BLOOD WHEN 5 CC. OF TUNGSTIC ACID FILTRATE IS USED**

Temp. ° C.	Mgm. Urea N. per 100 c.c. When Gas Volume Is		Mgm. Urea per 100 c.c. When Gas Volume Is	
	0.5 c.c.	2 c.c.	0.5 c.c.	2 c.c.
15 .....	0.1561	0.624	0.335	1.336
16 .....	55	22	34	31
17 .....	49	20	32	26
18 .....	44	18	31	22
19 .....	38	15	30	17
20 .....	33	13	29	13
21 .....	27	11	28	08
22 .....	22	09	27	03
23 .....	16	06	25	1.298
24 .....	11	04	24	94
25 .....	06	02	23	90
26 .....	00	00	22	85
27 .....	0.1495	0.598	21	80
28 .....	90	96	20	76
29 .....	85	94	19	72
30 .....	80	92	18	67
31 .....	74	90	16	62
32 .....	69	88	15	58
33 .....	64	86	14	54
34 .....	59	84	13	50

### **MOLLER, McINTOSH AND VAN SLYKE'S UREA CLEARANCE TEST**

**Principle.**—In this method (*Jour. Clin. Inv.*, 6: 427, 1928) the excretory efficiency of the kidneys is measured by the amount of blood cleared of urea in 1 minute as determined by the ratio of the blood urea to the amount of urea excreted in the urine during a fixed time.

**Reagents.**—These are the same as used in the determinations of urea in blood and urine.

**Procedure.**—1. About 30 minutes prior to the beginning of the test, the subject is given 2 glasses of water.

2. The bladder is emptied completely, the urine discarded and the exact time noted.

3. About 1 hour later, the subject again completely empties the bladder. Note the time since the previous voiding exactly, measure the urine volume in cc., and store in the refrigerator.

4. Remove an amount of blood, in the usual manner, sufficient for a urea determination.

5. About 1 hour after the previous voiding the bladder is again completely emptied. Note the time since the previous voiding and measure the urine volume in cc.

6. Determine the urea nitrogen content per 100 cc. of both urine samples and the blood sample.

**Calculations.**—1. Divide each urine volume by its respective time in minutes. This is the *minute volume* and 2 different formulae are used, depending upon whether this value is more or less than 2 cc. per minute.

2. If the urine volume is over 2 cc. per minute then the formula for *maximum clearance* is used:  $\frac{U \text{ (urine urea N)}}{B \text{ (blood urea N)}} \times V \text{ (urine cc. per min.)} = \text{the cc. of blood cleared of urea per minute.}$  This volume has been found to have a normal average value of 75 cc. Therefore, the per cent of *average normal function* is  $\frac{100}{75} \times \frac{U}{B} \times V$  or  $\frac{1.33UV}{B}$ . When the values found above are substituted in this formula the result is per cent of *average normal function* for maximum clearance.

3. If the urine volume per minute is 2 cc. or less, then the square root formula for *standard clearance* is used:  $\frac{U}{B} \times \sqrt{V} = \text{the cc. of blood cleared of urea per minute.}$  This volume has been found to have a normal average of 54 cc. Therefore the per cent of *average normal function* is  $\frac{100}{54} \times \frac{U}{B} \times \sqrt{V}$  or  $\frac{1.85U \sqrt{V}}{B}$ . To facilitate calculation, values for  $\sqrt{V}$  are appended in the table. Substituting the values found in this formula gives the per cent of *average normal function* for standard clearance.

V	$\sqrt{V}$	V	$\sqrt{V}$	V	$\sqrt{V}$	V	$\sqrt{V}$
0.2	0.45	0.7	0.84	1.2	1.1	1.7	1.3
0.3	0.55	0.8	0.89	1.3	1.14	1.8	1.34
0.4	0.63	0.9	0.95	1.4	1.18	1.9	1.38
0.5	0.71	1.0	1.00	1.5	1.23	2.0	1.42
0.6	0.78	1.1	1.05	1.6	1.27	2.1	1.45

**Notes.**—1. The use of the two 1-hour urine specimens is for the purpose of a check; however, when one urine specimen is large and the other small, duplicate results will not be obtained. In such a case it is best to combine the 2 urine specimens. Divide the urine volume thus obtained by the total elapsed time in order to obtain the volume per minute.

2. In applying either formula to children or persons differing markedly from usual adult size, the surface area of the subject should be determined from the height and weight, and the value "V" modified by multiplying it by the factor 1.73

square meters of surface area.

3. Inaccurate results are obtained if (a) the volume of urine is less than 20 cc. per hour, or (b) if the bladder is not completely emptied at each voiding. If incomplete emptying is suspected catheterization should be resorted to.

4. The normal range is 75 to 130 per cent of normal function with either formula. Values below 70 per cent are indicative of impaired renal function and such lowered values are obtained, as a rule, before evidence of urea retention in the blood is observed. Values as low as 10 per cent or less are found in the terminal stages of nephritis or uremic coma.

### KOCH AND McMEEKIN METHOD FOR DETERMINATION OF NONPROTEIN NITROGEN

**Principle.**—In this method (*Jour. Am. Chem. Soc.*, 46: 2066, 1924) the protein-free blood filtrate is treated with an acid mixture and peroxide which converts the nitrogen into ammonia. The solution is nesslerized and read against a standard ammonium sulfate solution similarly treated.

**Apparatus.**—One test tube, Pyrex, 75 cc. capacity, 25 x 200 mm., graduated at 35 and 50 cc.

Micro burner.

Retort stand with clamp.

**Reagents.**—1. *Sulfuric acid*, 50 per cent. Carefully mix equal parts of concentrated sulfuric acid and distilled water; cool.

2. *Hydrogen peroxide*, 30 per cent, nitrogen-free.

3. *Nessler's reagent*, for preparation see page 797.

4. *Standard Ammonium Sulfate Solution.*—Ammonium sulfate (C.P., special, pyridine free) should be dried in a hot air oven for  $\frac{1}{2}$  hour at 110° C. and then allowed to cool 20 minutes in a desiccator. Weigh on an analytical balance 0.142 gram. Wash into a beaker to dissolve, then wash into a liter volumetric flask. Add 1 cc. concentrated C.P. hydrochloric acid (to prevent growth of molds). Dilute to the mark with distilled water. Keep in a well stoppered bottle (5 cc. = 0.015 mg. of nitrogen).

**Procedure.**—1. Pipet 5 cc. of protein-free blood filtrate into a dry, lipped, thin-walled, 75 cc. Pyrex test tube (200 by 25 millimeters) graduated at 35 cc. and 50 cc.

2. Add 1 cc. of the sulphuric-water mixture and, to prevent bumping, add a dry Pyrex glass bead or a quartz pebble (may be omitted).

3. Clamp the test tube in a test tube support and boil vigorously with the micro-burner until the characteristic dense acid fumes fill the tube. This will occur in from 3 to 7 minutes, depending on the size of the flame.

4. Cool slightly and add 3 drops of 30 per cent hydrogen peroxide.

5. Turn down the flame so that the contents are just visibly boiling and, after fumes appear, close the mouth of the tube with a short stemmed funnel. Continue the heating for a few minutes or for 2 minutes after the digest is perfectly clear.

6. Turn out the flame, remove the funnel, and allow to cool.

7. When cooled to room temperature, add about 20 cc. of water, 2 drops of gum ghatti solution, and dilute to the 35 cc. mark.

8. Add Nessler's reagent to the 50 cc. mark, insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion, giving a crystal clear fluid above a white sediment (silica). If the sediment is colored, the nesslerization was not successful and the determination must be discarded. The unknown and the standard should be nesslerized at approximately the same time.

9. Prepare the standard as follows: Pipet 5 cc. of ammonium sulphate standard

into a 50 cc. volumetric flask. Add 1 cc. of the acid mixture. Add about 30 cc. of distilled water and 2 drops of gum ghatti solution. Add 15 cc. Nessler's reagent. Dilute to the mark and mix.

10. Compare the standard and unknown in the colorimeter after 10 minutes, the standard being set at 20.

**Calculation.**— $\frac{20}{R} \times 30 =$  milligrams nonprotein nitrogen per 100 cc. of blood.

Or: set the unknown at 15; then  $2 \times$  reading of standard = mg. per cent of nonprotein nitrogen in original blood.

*Notes.*—1. Photoelectric comparison is advantageous in this determination for the reasons given under the urea nitrogen determination. The procedure for the color development is exactly the same. Use filter No. 54 and compare against a distilled water zero. The blank should contain all the reagents except the blood filtrate. As in the urea determination the blank reading, which should be subtracted from the unknown and standard readings, will be found to be small and constant for any given set of reagents and it and the standard reading need only be determined at intervals.

Calculation:  $\frac{30}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{mg. per cent of nonprotein nitrogen in original blood.}$

2. In the digestion, bumping is often a source of serious difficulty. The most important cause of bumping lies in the condition of the test tube. In dry test tubes and beads the very fine pores are filled with air and until this air has been driven out by heat, localized formation of steam occurs and the boiling is smooth and even, but as these pores are gradually filled with the liquid bumping begins.

3. In case of bumping after repeated determinations, heat the tube to red heat in a flame, cool and rinse with alcohol.

4. The amount of nonprotein nitrogen in normal blood ranges from 25 to 35 milligrams per 100 cc.

5. The nitrogen estimated by this method represents the nitrogen of blood constituents which are not thrown down by the precipitant but remain in solution. This nitrogen has been called the "nonprotein nitrogen" and "uncoagulable nitrogen". Of the total nitrogen of the blood, it is about 1 per cent. Its principally known constituents are urea, uric acid, creatinine, creatine and amino acids. The nitrogen in these does not equal the total nonprotein nitrogen. The difference has been called the "undetermined nitrogen" and contains principally peptid and peptone nitrogen. Urea nitrogen represents from 40 to 65 per cent of the total, with a normal average of 50 per cent of the total nonprotein nitrogen. Findings higher than 40 milligrams indicate nitrogenous retention; the failure of the kidney to eliminate waste products. By determining the amounts of the known constituents, particularly urea, uric acid and creatinine, more detailed information is obtained than by the determination of the total nonprotein nitrogen only.

6. In the case of bloods containing excessive amounts of nonprotein nitrogen a cloudiness results; the determination should be repeated, using 2 or 1 cc. portions of filtrate.

7. A slightly excessive concentration of alkali at once precipitates the colloidal colored ammonium compound. Local zones of excessive alkalinity coagulate part of

the solution. The Nessler reagent is dilute enough so that localized zones of alkalinity ordinarily do not occur.

8. Turbidity in the final solution, aside from that due to silicon dioxide, is ordinarily due to the fact that the Nessler's solution is too strongly alkaline or the acid digestion mixture is too weak or too much sulphuric acid has been lost during digestion.

9. The use of funnels is primarily to prevent loss of sulphuric acid so that the alkalinity of the nesslerized standard and unknown shall be the same. The greater the alkalinity the deeper is the color. In eliminating the loss of sulphuric acid fumes there is also eliminated all danger of losing ammonia together with those fumes.

10. The use of sulfuric acid-water plus 30 per cent peroxide permits rapid digestion without the corrosion of the glass tube which takes place when phosphoric acid is used in the digestion mixture.

### FRAME, RUSSEL AND WILHELMI METHOD FOR DETERMINATION OF AMINO ACID NITROGEN

**Principle.**—This method (*Jour. Biol. Chem.* 149: 255, 1943) depends upon the combination of amino nitrogen groups with beta naphthoquinone sulfonate to form highly colored compounds which may be measured colorimetrically.

**Reagents.**—1. *Sodium beta-naphthoquinone-4-sulfonate* (Eastman), 0.5 per cent solution, freshly prepared.

2. *Sodium tetraborate* (borax), 1 per cent solution.

3. *Sodium hydroxide*, N/10.

4. *Acid-formaldehyde*: 3 parts of approximately 1.5 N hydrochloric acid, 1 part of glacial acetic acid, and 4 parts of formaldehyde (made by diluting 11.3 cc. of 40 per cent formaldehyde to 1 liter).

5. *Sodium thiosulfate*, N/10.

6. *Stock Standard Amino Acid Solution*: 0.268 gm. of glycine and 0.525 gm. of glutamic acid dissolved in exactly 500 cc. of a solution of 0.2 per cent sodium benzoate in N/10 hydrochloric acid. It contains 0.2 mg. of amino nitrogen per cc. and keeps indefinitely.

7. *Working Standard Amino Acid Solution*: Just before use, 5 cc. of stock standard are diluted to 200 cc. with water (1.0 cc. = 0.005 mg. amino acid nitrogen).

**Procedure.**—1. Place 5 cc. of 1:10 tungstic acid filtrate into a test tube graduated at 15 cc.

2. Place 5 cc. of working standard into a similar tube.

3. Add 1 drop of 0.5 per cent alcoholic phenolphthalein solution to each tube.

4. To each tube add N/10 sodium hydroxide drop by drop until a permanent pink color is established.

5. Add 1 cc. of borax solution to each tube.

6. Add 1 cc. of the naphthoquinone solution to each tube, mix by sidewise shaking, and place in a briskly boiling water bath for 10 minutes.

7. Place the tubes in cold water for 5 minutes.

8. Add to each tube 1 cc. of the acid formaldehyde, mix, and then add 1 cc. of the N/10 thiosulfate.

9. Make up to the 15 cc. mark with water, mix, and let stand 10 minutes before reading in the colorimeter.

10. If a photoelectric colorimeter is used prepare a blank containing all reagents as above and distilled water in place of filtrate.

**Calculations:** 1. For visual colorimetry set the standard at 20, in which case  $\frac{100}{R} = \text{mg. per cent of amino acid nitrogen in original blood.}$

2. For photoelectric colorimetry use filter No. 49 and a water zero. Subtract the blank reading from the readings of both standard and unknown, in which case:

$\frac{5}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{mg. per cent of amino acid nitrogen in original blood.}$

**Notes.**—1. The normal amino acid nitrogen content of whole blood is 4 to 8 mg. per 100 cc.

2. Amino acids increase in leukemia and in acute yellow atrophy of the liver due to tissue autolysis.

3. Insulin will reduce the amino acid content of blood to almost as great a degree as blood sugar.

4. Since aromatic amines react with the quinone reagent, the presence in blood of unconjugated sulfonamide derivatives will increase the color effect slightly, *i.e.*, 10 mg. per cent of circulating sulfanilamide is equivalent to 0.8 mg. per cent of amino acid nitrogen. Arsphenamine, neoarsphenamine and mepharsen also have a slight enhancing effect.

5. The principle naturally occurring substances which might lead to erroneous results are ammonia, uric acid and allantoin, of which only uric acid is of importance in human blood. Its effect is to yield color equivalent to 0.1 mg. per cent of amino nitrogen when present in a concentration of 1.0 mg. per cent and may be disregarded unless the uric acid is very high.

## FOLIN AND WU METHOD FOR DETERMINATION OF CREATININE

**Principle.**—This method (*Jour. Biol. Chem.* 38: 81, 1919) depends upon comparing the yellow color produced in a protein-free blood filtrate by the action of alkaline picrate with the color similarly produced in a known standard solution of creatinine.

**Reagents.**—1. *Stock Creatinine Solution.*—In a 100 cc. volumetric flask dissolve 0.1 gram of creatinine in N/10 hydrochloric acid and dilute to the mark with the same acid.

2. *Standard Creatinine Solution.*—Pipet 3 cc. of the stock creatinine solution into a 500 cc. volumetric flask, add 100 cc. of N/10 hydrochloric acid and dilute to mark with water (5 cc. contain 0.03 milligram of creatinine).

3. *Hydrochloric Acid* (approximately N/10).—Dilute 10 cc. of hydrochloric acid to 1 liter.

4. *Saturated Picric Acid Solution.*—Place about 15 grams of purified picric acid in a large Erlenmeyer flask; add 1 liter of water; heat over low flame until the picric acid is dissolved. Cool and keep in dark. Decant the clear solution for use.

5. 10 per cent *Sodium Hydroxide Solution.*

6. *Alkaline Picrate.*—To 25 cc. of the saturated picric acid solution add 5 cc. of sodium hydroxide solution. This should be freshly prepared for each determination.



**Procedure.**—1. Pipet 10 cc. of protein-free blood filtrate into flask marked *B*.

2. Pipet 5 cc. of the standard creatinine solution into a second flask, marked *S*, and add 15 cc. of water.

3. Add 5 cc. of the alkaline picrate solution to flask *B*, and 10 cc. to flask *S*.

4. Mix each and let stand for 10 minutes. Compare in the colorimeter.

**Calculation:**

$S$  = reading of standard

$B$  = reading of blood filtrate

$x$  = milligrams of creatinine per 100 cc. of blood or

$$x = \frac{15 S}{B} = \frac{S}{10} \text{ if blood filtrate is set at 15 mm.}$$

**Notes.**—1. The normal range of creatinine is from 1 to 2 milligrams per 100 cc. of blood.

2. Five cc. of the standard gives a color for accurate colorimetric comparison with filtrates whose creatinine content is not over 2 milligrams per 100 cc. of blood. For bloods known or thought to contain values higher than this, use 10, 15, or 20 cc. of the standard with proportionally less water. If the content be very high, use less filtrate with proper dilution. Before the addition of the alkaline picrate the volume of *S* should be 20 cc. and the volume of the flask *B* should be 10 cc.

3. The yellow to yellowish brown colors developed in this method are very difficult to match accurately in a visual colorimeter, but are readily measured photoelectrically. The reagents are the same as in the method for visual colorimetry and the procedure is somewhat simpler:

**Unknown.**—To 5 cc. of 1:10 protein-free blood filtrate add 2.5 cc. of alkaline picrate solution. Mix and read in the colorimeter after 10 minutes against the *blank* tube zero setting, using a No. 54 light filter.

**Blank.**—To 5 cc. of distilled water add 2.5 cc. of alkaline picrate solution. Mix and place in colorimeter and adjust to zero.

**Standard.**—To 5 cc. of the creatinine solution containing 0.03 mg. of creatinine add 2.5 cc. of alkaline picrate solution. Mix and read in colorimeter after 10 minutes against the blank tube zero setting.

**Calculation.**— $\frac{6}{\text{reading of standard}}$  (= factor)  $\times$  reading of unknown = mg. per cent of creatinine in original blood.

4. The calibration factor determined in duplicate or triplicate is valid indefinitely and thus standard readings need only be made occasionally to insure that it has remained constant.

5. Since the proportionality is good from 0 to 10 mg. per cent, only the one standard need be set up if the photoelectric method is used.

6. The amount of creatinine is very constant for the individual and is not appreciably affected by diet, being almost entirely endogenous in origin. It is the last of the nitrogenous waste products to accumulate in nitrogenous retention. Hence its accumulation in the blood is of grave prognostic significance. In chronic conditions, once it begins to accumulate, it rarely decreases. A concentration of 5 milligrams or over per 100 cc. of blood is usually followed by death within a short period. In acute conditions and acute exacerbations of chronic conditions, the accumulation decreases

with the subsiding of the acute stage but when there has been a resulting damage to the kidney, the level will remain slightly above normal. The chief value of its determination is during the later stages of kidney disease.

### BROWN METHOD FOR DETERMINATION OF URIC ACID

**Principle.**—In this method (*Jour. Biol. Chem.* 68: 123, 1926) the color produced by the action of a phosphotungstic acid reagent with the uric acid in protein-free blood filtrate is compared with the color produced by the same reagent with a standard solution of uric acid.

**Reagents.**—1. *Stock Standard Uric Acid Solution.*—Weigh on an analytical balance 1 gram of uric acid and transfer to a funnel on a 300 cc. flask. Place 0.45 to 0.50 gram lithium carbonate in a beaker in about 150 cc. of water and heat to 60° C., stirring until all the carbonate has dissolved. With the hot carbonate solution, rinse the uric acid into the flask and shake. As soon as a clear solution is obtained, cool under running water, with shaking, and transfer to a volumetric liter flask. Rinse and dilute to a volume of 400 to 500 cc. Add 20 cc. of formalin, and after shaking to insure thorough mixing, add 3 cc. glacial acetic acid. Shake to remove most of the carbonic acid and dilute to the mark. Keep in small, tightly stoppered bottles in a dark place. This solution keeps indefinitely.

2. *Working Standard Uric Acid Solution.*—Dilute 1 cc. of the stock solution in a 200 cc. volumetric flask to the mark with water (5 cc. are equivalent to 0.025 milligram uric acid). This keeps about 1 week in the refrigerator.

3. *Sodium Cyanide Solution.*—In a graduated, glass-stoppered cylinder, dissolve 10 grams of sodium cyanide. Dilute to 200 cc. Prepare fresh each month.

4. *Uric Acid Reagent.*—Place in a 1000 cc. Erlenmeyer flask, 100 grams of molybdenum-free sodium tungstate, 70 cc. of phosphoric acid, and about 700 cc. of water. Boil gently for 2 hours using a reflux condenser. Allow to cool and dilute to 1 liter.

**Procedure.**—1. Pipet 10 cc. of protein-free blood filtrate into a 100 cc. Erlenmeyer flask marked *B*; add 5 cc. of water.

2. To two other flasks marked *SI* and *SII* add, respectively, 5 and 10 cc. of the uric acid standard, and 10 and 5 cc. of water.

3. To all flasks add, from buret, 5 cc. of sodium cyanide solution. Then add 0.5 cc. of the uric acid reagent.

4. Mix.

5. Allow to stand 20 minutes.

6. Compare in colorimeter the solution in flask *B* with that standard which it appears, by inspection, to more nearly to resemble in color.

#### Calculation:

*SI* = reading of standard I

*SII* = reading of standard II

*B* = reading of blood filtrate

*x* = milligrams of uric acid in 100 cc. of blood

$$x = \frac{2.5 \text{ SI}}{B} \text{ or } x = \frac{5 \text{ SII}}{B}$$

Set blood filtrate at 10 mm. when  $x \frac{SI}{4}$

Set blood filtrate at 10 mm. when  $x \frac{SII}{2}$

*Notes.*—1. The disturbing effect of possible clouding during the 20-minute waiting period can be avoided if sodium and not potassium oxalate is used as anticoagulant.

2. It has been found that the formaldehyde formerly used as a preservative in the diluted working standard sometimes causes non-specific chromogenic effects. It is therefore directed that this solution be made up fresh by a simple 1-200 dilution of the stock standard.

3. The normal range of uric acid is from 2 to 4 milligrams per 100 cc. of blood.

4. Uric acid is the end-product of purin metabolism, and is partly exogenous and partly endogenous.

5. It is increased in kidney dysfunction, but has no clinical value herewith which is not indicated by the urea nitrogen determination.

6. It is increased, with little or no increase in the other nitrogenous constituents in gout, and cardiac decompensation.

### FOLIN AND WU METHOD FOR DETERMINATION OF GLUCOSE

**Principle.**—This method (*Jour. Biol. Chem.* 41: 367, 1920) is based upon the principle that when protein-free blood filtrate is heated with an alkaline copper solution, the glucose produces a precipitate of cuprous oxide which is dissolved by and reduces a phosphomolybdic acid solution. The resulting blue color is compared colorimetrically with one similarly prepared from a standard glucose solution.

**Reagents.**—1. *Alkaline Copper Solution.*—Dissolve 40 grams of sodium carbonate in 400 cc. of water. Separately dissolve 7.5 grams of tartaric acid in 300 cc. of water. Separately dissolve 4.5 grams of cupric sulphate in 200 cc. of water. In each case use heat to hasten solution. Cool to room temperature and pour the tartaric acid solution into the carbonate solution, then add the cupric sulphate solution. Dilute to 1 liter. Mix. A red sediment may form in the course of 1 or 2 weeks. If this happens, pour off the clear supernatant reagent or filter through a good quality filter paper. This reagent keeps indefinitely.

2. *Phosphomolybdic Acid Solution.*—Dissolve 40 grams of sodium hydroxide in 400 cc. of water. Add 70 grams of molybdic acid, 10 grams of sodium tungstate and 400 cc. of water. Boil for  $\frac{1}{2}$  hour. Cool. Dilute to 700 cc. Add 250 cc. of phosphoric acid and dilute to 1 liter. Mix.

3. *Benzoic Acid.*—Dissolve 2.5 grams of benzoic acid in 1 liter of hot water and cool. Transfer to a bottle; the solution will keep indefinitely. Filter as necessary.

4. *Standard Sugar Solutions.*—**Stock.**—Weigh 1 gram of pure glucose (Bureau of Standards sample No. 41) on an analytical balance. Transfer to a 100 cc. volumetric flask, dissolve, and fill to the mark with benzoic acid solution. This 1 per cent stock solution keeps indefinitely.

**SI. STANDARD CONTAINING 10 MILLIGRAMS DEXTROSE PER 100 cc.**—Pipet 5 cc. of stock solution into a 500 cc. volumetric flask and dilute to the mark with benzoic acid solution.

## FOLIN AND WU METHOD FOR DETERMINATION OF GLUCOSE 811

**SII. STANDARD CONTAINING 20 MILLIGRAMS DEXTROSE PER 100 cc.**—Pipet 10 cc. of stock solution into a 500 cc. volumetric flask and dilute to the mark with benzoic acid solution.

Standards SI and SII will keep at least 6 months.

**Procedure.**—1. Pipet (Fig. 321) 2 cc. of protein-free blood filtrate into a special Folin tube (Fig. 322). Label B.



FIG. 321.—FOLIN-WU PIPET

2. To a similar tube, add 2 cc. of standard sugar solution SI. Label SI.
3. To a third Folin sugar tube add 2 cc. of standard sugar solution SII. Label SII.
4. To each tube add 2 cc. of the alkaline copper solution.
5. Transfer the tubes to a boiling water bath and heat for 8 minutes.
6. Cool for 2 to 3 minutes in a cold water bath without shaking.
7. Add to each tube 2 cc. of the phospho-molybdic acid solution.
8. Let stand for 3 minutes: dilute the resulting solution to the 25 cc. mark.
9. Insert a rubber stopper and mix. (It is essential that adequate attention be given to the mixing because the greater part of the blue color is formed in the bulb of the tube.)
10. After 10 minutes compare in a colorimeter with the nearest matching standard.

### Calculation:

$B$  = reading of blood sample.

$$x = \frac{100 \text{ SI}}{B} \text{ or } x = \frac{200 \text{ SII}}{B}$$

When using standard SI set the blood sample at 20 mm., when  $x = 5 \times \text{SI}$ .

Or, when using standard SII set the blood sample at 10 mm., when  $x = 20 \times \text{SII}$ .

The color comparison may be made in the photoelectric colorimeter using the above method exactly, if light filter No. 42 is used. Unlike visual colorimetry, the colors are found to be strictly proportional to the concentration up to about 400 mg. per cent and only one standard need be prepared. Comparison should be made against a water zero setting. Both the reagent blank reading as well as the standard reading remain constant over an indefinite period and need only be checked when new reagents are prepared. If standard SII be used, the calculation is:

$$\frac{200}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{mg. per cent of reducing substances in original blood.}$$

**Notes.**—1. Complete cooling of the alkaline cuprous oxide suspension before adding the phosphate molybdate solution is not essential. The important point is that the standard and the unknown should not only be heated the same length of time, but should also have approximately the same temperature when the acid reagent is added.



FIG. 322.—FOLIN BLOOD SUGAR TUBE

2. The normal range of concentration of blood glucose for the fasting adult is from 80 to 110 milligrams per 100 cc. of blood.'

3. If the blood filtrate gives a color too deep for accurate colorimetric comparison with the stronger standard, the test is repeated, substituting for the 2 cc. of blood filtrate in tube B, 1 cc. of blood filtrate and 1 cc. of distilled water. The final result must therefore be doubled.

4. Glucose determinations are made immediately after taking the blood sample as the glucose rapidly disappears by glycolysis. Efficient refrigeration retards, but does not prevent, glycolysis.

5. When the analysis cannot be made immediately, the proteins of the blood should be precipitated and the filtrate, to which are added a few drops of toluene, placed in the refrigerator. This filtrate will give accurate readings for 24 hours.

6. A rise in blood glucose follows absorption from the intestinal tract. The fasting level is again reached within 3 hours after the ingestion of food. In some pathologic conditions, *e.g.*, diabetes mellitus, this return is delayed and the rise is higher than in normals. This forms the basis of glucose tolerance tests.

7. Hyperglycemia is found in diabetes mellitus, some cases of advanced nephritis, and frequently in emotional states.

8. Hypoglycemia has been reported after thyroidectomy and in some hypoendocrine conditions.

9. One determination of the blood glucose is not sufficient on which to base a diagnosis of hyperglycemia; the high level must be constant.

10. In "renal glycosuria" there is no hyperglycemia although there is glycosuria.

11. Glycosuria (the presence of glucose in the urine when examined by usual laboratory tests) is not dependent directly on the level of the blood glucose. The point of concentration which when reached results in glycosuria has been called the "renal threshold." This appears to be individual and not a definite point; for most normal persons concentrations of 160 to 180 milligrams result in glycosuria. These figures are higher in diabetes mellitus and late nephritis, but lower in "renal glycosuria." The significance of glycosuria cannot be determined without simultaneous blood glucose determinations.

12. The glucose content of the blood as usually determined is not chemically true as it is actually a measurement of copper reducing substances. The glucose in normal blood is approximately 90 per cent of the copper reducing material. As the physician has been taught to use normals derived from copper reducing substances it is possibly safer to retain this line of thought.

### FOLIN AND MALMROS MICROMETHOD FOR DETERMINATION OF GLUCOSE

**Principle.**—In this method (*New England Jour. Med.* 206: 727, 1932) when a glucose solution is heated with an alkaline ferricyanide solution, the ferricyanide is reduced to ferrocyanide. The blue color produced when ferrocyanide reacts with a ferric iron solution is measured in a colorimeter.

**Reagents.**—1. *Sulfate-Tungstate Solution.*—To about 250 cc. of water in a 500 cc. volumetric flask add 10 gm. of anhydrous sodium sulfate and 15 cc. of 10 per cent sodium tungstate solution. Shake to dissolve and dilute to the mark.

2. *Sulfuric Acid Solution*.—Place 96 cc. of N/12 sulfuric acid in a 100 cc. graduated cylinder. Add 2 gm. of anhydrous sodium sulfate. Stopper and shake until dissolved. Add water to the 100 cc. mark and mix.

3. *Potassium Ferricyanide Solution*.—Dissolve 2 grams of C.P. potassium ferricyanide in distilled water and dilute to 500 cc. The major part of this solution should be kept in a brown bottle in a dark closet. The reagent in daily use should also be kept in a brown bottle. Prepare anew when it gives an appreciable blue colored blank with the ferric iron solution.

4. *Sodium Cyanide-Carbonate Solution*.—Transfer 8 grams of anhydrous sodium carbonate to a 500 cc. volumetric flask. Add 40 to 50 cc. of water and shake, to promote rapid solution. Add 150 cc. of freshly prepared 1 per cent sodium cyanide solution; dilute to volume and mix.

5. *Ferric Iron Solution*.—To one liter of gum ghatti solution add a solution of 5 grams of anhydrous ferric sulfate in 75 cc. of 85 per cent phosphoric acid plus 100 cc. of water. Add to the mixture, a little at a time, 1 per cent potassium permanganate solution until a pink color remains which persists for at least 30 minutes. The solution keeps indefinitely.

6. *Standard Glucose Solution*.—To a liter volumetric flask add 1 cc. of stock (1 per cent) glucose solution; add 125 cc. of 0.2 per cent benzoic acid; dilute to the mark and mix (1 cc. = 0.01 mg. of glucose).

7. *Picrate Light Filter*.—A special glass light filter may be purchased or a filter may be prepared as follows: Dissolve 5 grams of picric acid in 100 cc. of methyl alcohol and add 5 cc. of 10 per cent sodium hydroxide solution. Place a pack of 8 to 10 filter papers (of the correct size to cover colorimeter lamp) on a level and smooth mat of newspapers. Pour the acid picrate solution on the filters until the papers are saturated and an excess of solution which filters through at the bottom flows out a distance of at least 2 centimeters on the newspaper mat. When all the liquid has evaporated and the filter papers are perfectly dry, pour over the pack an excess of a 3 per cent solution of paraffin in benzine (gasoline) and again let the papers dry. A heavy filter with good absorbing qualities is best.

These picrate light filters may be placed over the opening in the colorimeter light, but it is also satisfactory to cut them into pieces which may be fitted over the reflector of the colorimeter. The reflection from the yellow paper is sufficient for good color matching.

**Procedure.**—1. In a 15 cc. conical centrifuge tube place 3.9 cc. of the sulfate-tungstate solution.

2. With a special 0.1 cc. Folin micro blood pipet, collect 0.1 cc. of blood from an ear or finger and transfer it to the solution in the centrifuge tube, rinsing with the solution 2 or 3 times.

3. Add 1 cc. of the sulfuric acid solution, mix by stirring and centrifuge.

4. Transfer 2 cc. of the supernatant fluid to a test tube graduated at 25 cc. and add 2 cc. of water.

5. Transfer 4 cc. of the standard sugar solution to a similar tube.

6. To each tube add 2 cc. of the potassium ferricyanide solution.

7. To each add 1 cc. of the sodium cyanide-carbonate solution.

8. Heat in boiling water for 8 minutes and cool in running water for 2 minutes.

9. To each add 5 cc. of the ferric iron solution and mix.

10. Allow to stand for 2 minutes and dilute to the 25 cc. graduation.
11. Using the picrate light filter, adjust the colorimeter so that the 2 fields have the same color intensity. Compare the 2 solutions after 10 minutes.

Colorimeter readings between 40 and 5 millimeters may be accepted without repeating the determination.

**Calculations:**  $X =$  milligrams of glucose in 100 cc. blood

$S =$  reading of the standard

Set the blood filtrate at 20 mm. when  $X = 5 \times S$

Color comparisons may be made in the photoelectric colorimeter. Use filter No. 54 and a distilled water zero setting. Make a reagent blank reading and subtract from the readings of standard and unknown. If the standard is of the strength used for visual colorimetry the calculation is:  $\frac{100}{\text{reading of standard}} (= \text{factor}) \times \text{the reading of the unknown} = \text{mg. per cent of glucose in original blood.}$

*Notes.*—1. This method is recommended for use in cases where it is difficult to do a venipuncture or when the patient must be bled frequently.

2. Blood taken from the finger is a mixture of venous and arterial blood and the sugar values after a glucose meal are higher than corresponding ones from venous blood alone.

3. The picrate filters are to eliminate the yellow color of the ferricyanide solution. When the colors are nearly the same, the filter may be dispensed with.

### METHODS FOR DETERMINATION OF GLUCOSE TOLERANCE

1. Breakfast is omitted and the test conducted after fasting overnight.
2. Urine (No. 1) and blood (No. 1) are taken for sugar determinations.
3. Immediately thereafter give by mouth 1.75 grams of glucose per kilogram of body weight dissolved in 500 cc. of water; flavor with lemon juice and cool with ice.
4. One hour later take urine (No. 2) and blood (No. 2) for sugar determinations.
5. One hour later (2 hours after taking the glucose), take urine (No. 3) and blood (No. 3).
6. One hour later (3 hours after taking the glucose), take urine (No. 4) and blood (No. 4).
7. As a general rule these are sufficient although it is sometimes advisable to take another specimen of urine (No. 5) and blood (No. 5) 1 hour later (4 hours after taking the glucose).
8. Conduct glucose determinations on all samples of blood and urine. Plot the blood glucose determinations in a curve against time on plain cross-section paper.
9. Normally the blood glucose rises to about 160 milligrams per 100 cc. at the end of the first hour, with no sugar in the urine, reaching the normal fasting level at the end of 2 to 3 hours.
10. In diabetes mellitus the blood glucose rises above 170 milligrams per 100 cc. with sugar in the urine and does not reach the fasting level until after 3 or 4 hours.
11. In "renal glycosuria" the blood glucose rises with large amounts in the urine but declines rapidly, reaching the fasting level in 3 hours.
12. A glucose tolerance test should not be conducted if diabetes mellitus in an advanced state is known to be present.

**One-Hour Two-Dose Glucose Tolerance Test** (*Exton and Rose*).—1. Collect fasting blood and urine samples, No. 1.

2. Give patient by mouth first dose of glucose (50 grams dissolved in 325 cc. of water). Allow 1 to 2 minutes for its ingestion.

3. Thirty minutes after ingestion of glucose, collect blood and urine samples, No. 2.

4. Give patient second dose of glucose same as above.

5. Thirty minutes after ingestion of glucose collect blood and urine samples, No. 3.

6. Make blood and urine examinations in the usual manner.

*Notes*.—1. This tolerance has the advantage over the 5-hour older method in requiring less time and fewer samples of blood and urine. Some metabolists claim it is more specific than the older method. In the absence of endocrine disturbance it is more sensitive than the longer test for the detection of early true diabetes.

2. A normal tolerance gives a normal fasting No. 1 blood glucose and a negative No. 1 urine sugar. Blood No. 2 shows a rise not exceeding 75 milligrams and a negative No. 2 urine sugar. Blood No. 3 is less, the same or does not exceed the sugar content of No. 2 by more than 5 milligrams and a negative No. 3 urine sugar.

3. In diabetes the blood glucose of No. 3 is 10 or more milligrams higher than No. 2. The urines may or may not be positive for sugar according to the severity of the disease.

4. Should the No. 3 blood glucose rise sufficiently to classify the tolerance as being abnormal and all the urines are negative, then a fourth sample of urine may be collected 1 hour after No. 3 which in most cases will be positive for glucose.

## VAN SLYKE AND CULLEN METHOD FOR DETERMINATION OF CARBON DIOXIDE CAPACITY OF PLASMA

**Principle**.—In this method (*Jour. Biol. Chem.*, 30: 289, 1917) blood plasma is shaken in a separatory funnel filled with an air mixture the carbon dioxide tension of which approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known volume of the saturated plasma is then run into a special apparatus (Fig. 323), acid is added and carbon dioxide is liberated by the production of a partial vacuum. The carbon dioxide is measured at atmospheric pressure and the volume corresponding to 100 cc. of plasma is calculated.

**Apparatus**.—Van Slyke vacuum pipet.

Barometer.

Separatory funnel with long stem.

**Reagent**.—*Lactic acid*: Dilute 5 cc. of lactic acid with water to 50 cc.

**Procedure**.—1. Collect approximately 5 cc. of blood from an arm vein in an oxalated test tube containing 1 cc. of paraffin oil. The stopper is loosened and the blood stirred with the inlet tube to assure mixing with the oxalate. The tube should not be shaken or inverted.

2. Remove the stopper and centrifuge the tube.

3. With a capillary pipet transfer the plasma to a separatory funnel. Putting the stem of the separatory funnel in the mouth pass the air of 3 complete exhalations



through the plasma. Close the stopper and cock of the funnel and rotate it for 3 minutes.

4. Rinse the cup at the top of the Van Slyke apparatus with water, and introduce into it 1 cc. of distilled water. Using a 1 cc. Mohr pipet introduce beneath the water 1 cc. of plasma. Add 1 or 2 drops of caprylic alcohol. Carefully draw the plasma, water and alcohol into the pipet until the mercury meniscus reaches the 2 cc. mark, the upper

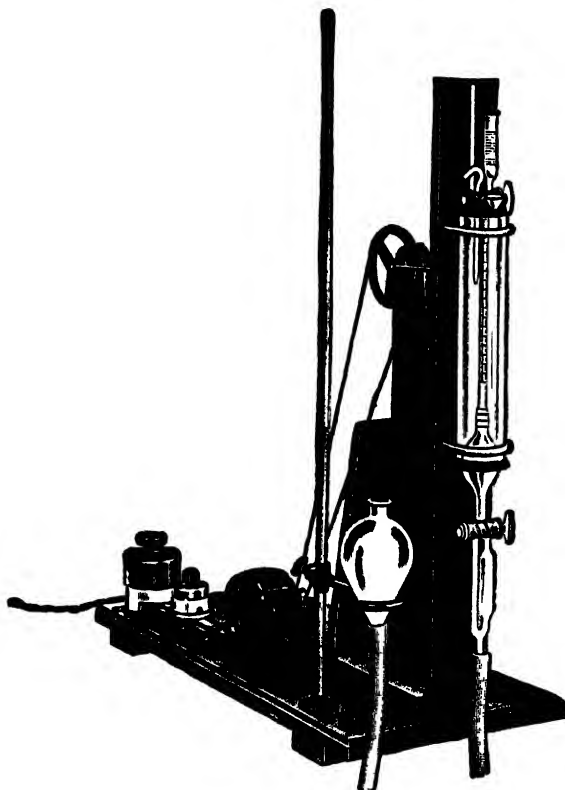


FIG. 323.—PRECISION MODEL OF THE VAN SLYKE GAS ANALYSIS APPARATUS WITH WATER JACKET AND STATIC SHAKER

meniscus of the solution being in the bottom of the cup of the pipet. Introduce about 1 cc. of lactic acid solution into the cup and draw enough into the pipet to bring the mercury meniscus to the 2.5 cc. mark. By lowering the reservoir, bring the mercury level to the 50 cc. mark and close the lower cock. Shake for 1 minute. Slowly and without oscillation reduce the gas to atmospheric pressure by raising the surface of the mercury in the reservoir to a height equal to 1/13th of the height of the water column above the mercury in the pipet. Read the volume.

**Calculation.**— $X$  = cc.  $\text{CO}_2$  bound as bicarbonate in 100 cc. plasma.

$V$  = vol. in cc. of gas in pipet.

$B$  = observed barometric pressure in mm.

$t$  = temperature of gas, centigrade.

$$X = \frac{B}{760} (100.8 - 0.27t) (V + 0.002t - 0.136)$$

For barometric pressures near 760 mm. and temperatures from 20° to 25° C. an approximate calculation suitable for all clinical work is:

- If V is 0.60 to 0.70 multiply by 100 and subtract 12.
- If V is 0.40 to 0.60 multiply by 100 and subtract 11.
- If V is 0.35 to 0.40 multiply by 100 and subtract 10.
- If V is 0.25 to 0.35 multiply by 100 and subtract 9.
- If V is 0.20 to 0.25 multiply by 100 and subtract 8.
- If V is 0.15 to 0.20 multiply by 100 and subtract 7.
- If V is 0.12 to 0.15 multiply by 100 and subtract 6.

*Notes.*—1. Normal range: In a normal resting adult the range is from 53 to 70 volumes per cent, and for normal infants about 10 volumes per cent lower. A result of from 53 to 40 volumes per cent shows a mild acidosis, generally without visible symptoms. In cases yielding from 40 to 31 volumes per cent symptoms may be apparent. Less than 31 volumes per cent indicates a severe acidosis.

2. When not in use, the pipet should be kept filled with distilled water.

3. Free carbonic acid is present in the body fluids in such concentration that it binds as bicarbonate all bases not bound by other acids; it therefore represents the excess of base which is left after all the nonvolatile acids have been neutralized and is available for neutralization of further acids. In this sense the bicarbonate constitutes the alkaline reserve of the body. Entrance of free acids reduces it to an extent proportional to the amount of the invading acids. Both in normal and pathologic metabolism, acids invade the blood and bind some of the alkali. Normally the kidneys are able to eliminate these acids while retaining the alkali; by this mechanism the body is able to excrete an acid urine from an alkaline blood.

4. Acidosis is a condition caused by acid retention sufficient to lower either the bicarbonate below normal or the pH of the blood below normal (*i.e.*, toward the acid side). In diabetic acidosis, the acid-base balance is disturbed by abnormal formation of nonvolatile acids (*e.g.*, aceto-acetic and beta-oxybutyric acids), while in nephritis, it is due to the failure of the kidney mechanism of elimination. In both cases the available alkali is bound by these acids reducing it in proportion to the amount of acid. Under nearly all circumstances in which the respiratory apparatus is not specifically affected, the quantity of carbonic acid is so regulated that a normal pH is satisfactorily maintained.

5. This method suffices for the study of such metabolic conditions as diabetes, nephritis, and marasmus, in which the acid-base disturbance is due to retention of nonvolatile acids while the respiratory control of the blood reaction is unaffected. This method is not adequate to cover conditions in which the respiratory control is so disturbed that the pH becomes abnormal, *e.g.*, in anesthesia.

6. In such cases a determination of the hydrogen ion concentration together with the carbon dioxide content of the venous blood is of greater advantage in determining the source of the disturbance of the acid-base balance.

7. It is desirable to keep the amount of caprylic alcohol small (about 0.02 cc.) as larger amounts may appreciably increase the results, because of the vapor tension of impurities which the alcohol may contain, and because it dissolves much more air per unit volume than does water.

8. Catch the water residue and mercury overflow in a flask. It requires only wash-

ing with water, drying with filter paper and straining through cloth or chamois skin to prepare the mercury for use again.

9. Practically the only source of difficulty with the determination is the entrance of air through the stop cocks. It is essential that both cocks should be properly greased and air tight (see below). It is also necessary that the cocks (especially the lower one) should be held in place by rubber bands so that they cannot be forced out by pressure of the mercury.

10. For thorough cleaning remove the rubber tubing and fill the apparatus by suction with *aqua regia* and let stand several hours.

11. If 1 cc. of plasma is not available, 0.5 cc. may be used, in which case the volume of distilled water and acid used is halved so that the total volume of water solution introduced is only 1.25 cc., and in the calculation the *observed* volume of gas is multiplied by 2.

12. The determination can be performed on whole blood.

13. As the gas is being brought to atmospheric pressure, the meniscus of water over the mercury should be raised slowly in the narrow part of the apparatus so that there be no oscillation of the column and resulting excessive reabsorption of carbon dioxide. With faulty technic, 0.01 to 0.02 cc. of carbon dioxide may be reabsorbed.

14. The results of the carbon dioxide capacity determination are expressed in "volumes per cent" and have reference to the number of cc. of carbon dioxide measured at 0° C. and 760 millimeters pressure, chemically bound as bicarbonate in 100 cc. of blood plasma.

15. The carbon dioxide capacity may also be determined by using the manometric apparatus. For details, see Van Slyke and Neil (*Jour. Biol. Chem.*, 61: 523, 1924).

**Stop Cock Lubricant for Van Slyke Apparatus.**—One part of pure, unvulcanized, para rubber gum finely divided is dissolved by the aid of heat in 5 parts of vaselin. Keep in small ointment jars.

A thin layer of vaselin is first uniformly applied to the cock and the latter is fitted and turned several times. The rubber lubricant is then applied in the same manner. In warm weather relatively little vaselin is used; in cold weather more is needed. The two lubricants used in this manner have proved more satisfactory than a single lubricant made by dissolving the rubber gum with larger amounts of vaselin.

## WHITEHORN METHOD FOR DETERMINATION OF CHLORIDE

**Principle.**—In this method (*Jour. Biol. Chem.*, 45: 449, 1921) the principle of the Volhard method is employed, that is, precipitation of silver chloride with silver nitrate and titration of the excess silver nitrate by means of thiocyanate, using ferric ammonium sulfate as an indicator.

**Apparatus.**—Micro buret, 5 or 10 cc. capacity, graduated in 0.02 cc. divisions.

**Reagents.**—**Silver Nitrate Solution.**—Weigh on an analytical balance 2.905 grams of silver nitrate. Transfer to a liter glass-stoppered volumetric flask. Dissolve in water and dilute to the graduation. Preserve in a glass-stoppered brown bottle. One cc. is equivalent to 1 milligram of sodium chloride.

**2. Ammonium Thiocyanate Solution.**—Dissolve about 1.5 grams of ammonium thiocyanate in 1 liter of water. Standardize this solution against the silver nitrate solution by using 10 cc. of water in place of blood filtrate and following the directions

## WHITEHORN METHOD FOR DETERMINATION OF CHLORIDE 819

under "Procedure" given below and adjusting the thiocyanate solution by the addition of water and retitration until 10 cc. of the thiocyanate solution is equivalent to 10 cc. of silver nitrate solution.

### 3. *Ferric Ammonium Sulfate Solution*, 6 per cent.

**Procedure.**—1. Pipet 10 cc. of protein-free blood filtrate into a 50 cc. porcelain casserole.

2. Add 10 cc. of silver nitrate solution and stir with a glass rod.

3. Add 5 cc. of concentrated nitric acid (using a graduated cylinder); stir.

4. Add 5 cc. of the ferric ammonium sulphate solution and stir.

5. Allow to stand 5 minutes, protected from strong light.

6. Titrate with the ammonium thiocyanate solution added from the buret until a definite salmon red color persists for 15 seconds notwithstanding constant stirring.

**Calculation:**  $t = \text{cc. of ammonium thiocyanate solution used.}$

$(10 - t) 100 = \text{milligrams of sodium chloride in 100 cc. of blood.}$

**Notes.**—1. The normal concentration of the chlorides, as sodium chloride, in whole blood ranges from 450 to 520 milligrams per 100 cc. In the serum or plasma, the concentration ranges from 570 to 620 milligrams per 100 cc.

2. The protein-free blood filtrate used in this method is prepared according to the method described on page 794.

3. Chlorides of the blood are increased in some cases of nephritis, and in some cardiac conditions; while low values have been observed in fevers, pneumonia, severe diabetes, and after the administration of diuretics.

4. The determination of blood chlorides is of practical value as an indication or contraindication for a salt-free diet.

5. All glassware used must have been washed with distilled water and all reagents must be halogen-free.

6. It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction. The use of nitric acid is the essential point in the procedure, for aside from preventing the precipitation of silver phosphate, it flocks out the silver chlorides with a consequent reduction of the surface exposed. As silver thiocyanate is more insoluble than silver nitrate, it is evident that the surface of silver chloride exposed must be made as small as possible in order to prevent reaction between silver chloride and ferric thiocyanate. The abundance of ferric ammonium sulphate used also retards this reaction by reducing the ionization of the latter. This also deepens the end-point color by preventing the ionization of the red salt,  $\text{Fe}(\text{CNS})_3$ , into yellow  $\text{Fe}$  and colorless  $\text{CNS}$  ions.

7. Mixture of tungstic acid and chloride brings down more silver than can be accounted for by chloride alone, but the possibility for error is avoided by carrying on the titration in the presence of the precipitate when all the silver which has not been precipitated by chloride is available for titration with thiocyanate.

8. Whitehorn's greatest deviations with the method were  $-1.3$  and  $+1.2$  per cent and the limit of error with his careful technic was therefore less than 1.5 per cent. He used volumetric flasks to make the 1:10 dilution. An error of 0.1 cc. in measurement of silver nitrate will cause about 4 per cent error in the final result and an error of 0.1 cc. in measurement of thiocyanate will result in about 2 per cent error.

9. The method is applicable to serum and plasma as well as whole blood, but loss of carbon dioxide must be prevented until plasma has been separated from corpuscles.

### SCHALES AND SCHALES METHOD FOR DETERMINATION OF CHLORIDE

**Principle.**—In this method (*Jour. Biol. Chem.*, 140: 879, 1941) the chloride is titrated with mercuric nitrate solution, excess of mercury being indicated by the violet blue color formed with diphenylcarbazone.

**Reagents.**—1. *Mercuric nitrate solution.*—Dissolve about 1.4 gm. of mercuric nitrate in about 500 cc. of water with the addition of 20 cc. of 2N nitric acid. Transfer to a 1-liter volumetric flask and dilute to the mark with water.

2. *Indicator:* Dissolve 100 mg. of diphenylcarbazone (Eastman) in 100 cc. of 95 per cent alcohol. Store in the dark in a refrigerator. It keeps about 2 months and a fresh solution should be prepared when its color becomes cherry-red.

3. *Chloride Standard:* Dissolve 0.5 gm. of dry, reagent brand sodium chloride in water in a 1-liter volumetric flask and dilute to the mark. (1 cc. = 0.5 mg. of sodium chloride.)

**Procedure.**—1. Pipet 2 cc. of the standard chloride solution into a 25 cc. Erlenmeyer flask, add 4 drops of indicator solution and then the mercuric nitrate solution, dropwise, from a microburet calibrated at 0.02 cc. intervals. The first drop in excess turns the clear and colorless solution an intense violet-blue.

2. Adjust the strength of the mercuric nitrate solution by the addition of water, acidulated with 20 cc. of 2N nitric acid per liter, so that 2 cc. will be equivalent to 2 cc. of the standard chloride solution. Check the strength by repeating the above titration.

3. Pipet 2 cc. of a 1:10 tungstic acid filtrate of whole blood or serum into a 25 cc. Erlenmeyer flask, add 4 drops of indicator solution and the mercuric nitrate solution dropwise from a microburet until the violet-blue color is obtained as above.

**Calculation.**— $250 \times$  cc. of mercuric nitrate used = chloride as sodium chloride per 100 cc. of blood.

**Notes.**—1. In preparing the mercuric nitrate solution the amount of nitric acid as specified should be used; otherwise, the end point will not be sharp.

2. The mercuric nitrate solution is stable; the value of the solution requires only occasional checking against the standard chloride solution.

3. The microburet should have a tip small enough to deliver 0.02 cc. per drop.

4. Errors inherent in all modifications of the Volhard method due to the presence of the silver chloride precipitate, and the fading of the end point, are eliminated in this procedure since the mercuric chloride is soluble and the color of the mercury-carbazone complex does not fade.

### FISKE AND SUBBAROW METHOD FOR DETERMINATION OF INORGANIC PHOSPHORUS

**Principle.**—In this method (*Jour. Biol. Chem.*, 66: 375, 1925) the blue color obtained by adding molybdic acid and a reducing agent to an inorganic phosphate solution is compared colorimetrically with a standard phosphate solution similarly treated.

**Reagents.**—Ten times normal sulphuric acid ( $10\text{ N} \cdot \text{H}_2\text{SO}_4$ ) prepared as follows: 450 cc. concentrated  $\text{H}_2\text{SO}_4$  added to 1300 cc. water.

**Molybdate No. 1.**—12.5 grams ammonium molybdate in 100 cc. of water. To a 500-cc. graduated cylinder add 250 cc.  $10 \cdot \text{H}_2\text{SO}_4$  and the molybdate solution; dilute to the mark and mix.

**Molybdate No. 2.**—Prepare as above adding only 150 cc. of acid.

**10 Per Cent Trichloroacetic Acid.**—100 grams diluted to 1000 cc. with water.

**15 Per Cent Sodium Bisulphite.**—75 grams diluted to 500 cc. Let stand 2 to 3 days to free of turbidity. Filter. Keep well stoppered.

**20 Per Cent Sodium Sulphite.**—Dissolve 20 grams in water and dilute to 100 cc. Filter. Keep well stoppered.

**0.25 Per Cent Aminonaphtholsulphonic Acid.**—Dissolve 0.5 gram of dry powder in 195 cc. of the sodium bisulphite solution. Add 5 cc. of the sulphite solution. Stopper and shake until dissolved. If bisulphite is old, cloudiness results and more sulphite is required; add in 1 cc. quantities, until clear, being careful not to add too much. This solution will keep at least 4 weeks.

**Standard Phosphate Solution.**—Dissolve 0.3509 gram of potassium dihydrogen phosphate in water and transfer quantitatively to a 1-liter volumetric flask. Add 10 cc. of  $10\text{N } \text{H}_2\text{SO}_4$  and dilute to the mark. Mix. The solution will keep indefinitely. 5 cc. = 0.4 mg. P.

**Procedure.**—1. To a 50 cc. Erlenmeyer flask add 8 cc. of the trichloroacetic acid solution and, while gently rotating, add 2 cc. of oxalated whole blood plasma or serum. Stopper the flask, shake vigorously and filter through an ashless filter paper.

2. Measure 5 cc. of the filtrate into a 10 cc. volumetric flask or graduated cylinder, add 1 cc. of molybdate No. 2 and mix.

3. Add 0.4 cc. of aminonaphtholsulphonic acid solution, dilute to 10 cc. and mix.

4. Transfer 5 cc. of the phosphate standard solution to a 100 cc. volumetric flask. Add approximately 60 cc. of water. Add 10 cc. of molybdate No. 1 solution and 4 cc. of aminonaphtholsulphonic acid solution. Mix after each reagent is added and dilute to the mark. Mix.

5. Compare in the colorimeter after 5 minutes.

**Calculation.**—When the cup of the unknown is set at 10 millimeters, the reading of the standard cup multiplied by 0.4 gives milligrams of inorganic phosphorus per 100 cc. of blood.

**Notes.**—1. The application of this method to photoelectric colorimetry requires a few slight changes. The standard phosphate solution is made more dilute and one-half as much filtrate is used. Place 5 cc. of the standard solution used in the visual colorimetric method into a 100 cc. volumetric flask, add 50 cc. of the trichloroacetic acid solution and dilute to the mark with water. Of this standard 5 cc. = 0.02 mg. of phosphorus.

Place 2.5 cc. of the filtrate as used for visual colorimetry in a 10 cc. cylinder and add 2.5 cc. of water. In another cylinder place 5 cc. of the diluted standard. To each cylinder then add 1 cc. of molybdate No. 2 and 0.4 cc. of the amino reagent. Dilute to 10 cc., mix and compare after 5 minutes, using light filter No. 66, against a distilled water zero setting. The blank is prepared from 2.5 cc. of the trichloroacetic acid solution, 1 cc. of molybdate No. 2, 0.4 cc. of the amino reagent and water to the 10 cc. mark. The blank reading changes but little during the life of the reagents and is useful

in correcting for extraneous color formations. The blank reading is subtracted from the reading of the standard and unknown.

The calculation is  $\frac{4}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown}$  equals milligram per cent of inorganic phosphorus in original blood.

Both blank and factor need only be determined occasionally as a check on their constancy as long as the same reagents are used.

2. In adults, inorganic phosphorus is 3 to 4 mg. per 100 cc. of serum. Children have a higher content, about 5 to 5.5 mg. per 100 cc. There is an increase of this phosphorus in cases of nephritis with acidosis, and a slight increase during bone repair after major fractures. In active rickets, the phosphorus is regularly reduced. In tetany, the phosphorus may be normal or reduced.

### LETONOFF AND REINHOLD METHOD FOR DETERMINATION OF INORGANIC SULFUR

**Principle.**—In this method (*Jour. Biol. Chem.*, 114: 147, 1936) proteins are precipitated by uranium acetate, thereby also removing phosphorus. The sulfur is precipitated in the filtrates by benzidine hydrochloride and the color produced by the reaction of B-naphthoquinone-4-sulfonate on the solution of the precipitate is compared with a standard benzidine solution similarly treated.

**Reagents.**—1. *Uranium acetate solution*: Dissolve 0.8 gm. of uranium acetate in 200 cc. of water.

2. *Benzidine solution*: A filtered 1 per cent solution in acetone. Store in the refrigerator in a brown bottle; it should be discarded when it becomes highly colored.

3. *Sodium hydroxide-sodium borate solution*: Dissolve 1.0 gm. of powdered sodium borate in 100 cc. of 0.1N sodium hydroxide.

4. *Sodium B-naphthoquinone-4-sulfonate solution*: Dissolve 0.15 gm. of the pure compound in 100 cc. of water. It keeps about 2 weeks in the refrigerator.

5. *Glacial acetic acid*.

6. *Acetone*, 99.5 per cent.

7. *Standard Benzidine Hydrochloride*.—The benzidine hydrochloride is first purified as follows: Dissolve 5 gm. of the compound in 200 cc. of 5 per cent hydrochloric acid by warming to about 50° C. Filter off any insoluble residue. To the filtrate add 20 cc. of concentrated hydrochloric acid with continuous stirring. Cool in ice water for about 30 minutes, then filter off the crystals that have formed on a Buchner funnel. Wash with cold diluted hydrochloric acid (15 cc. of the concentrated acid to 100 cc. of water) using suction to remove the acid, then twice with 25 cc. portions of cold alcohol and 4 times with ether. The dry crystals should always be stored in a brown bottle.

Transfer 0.1606 gm. of the purified benzidine hydrochloride to a 200 cc. volumetric flask, dissolve in water previously warmed to about 50° C., cool and dilute to the mark. Store the solution in the refrigerator (10 cc. is equivalent to 1.0 mg. of sulfur).

8. *Dilute Standard Benzidine Hydrochloride*.—Dilute 10 cc. of the above solution to the mark in a 100 cc. volumetric flask. Store in the refrigerator (1.0 cc. is equivalent to 0.01 mg. of sulfur).

**Procedure.**—1. Pipet 6.0 cc. of the uranium acetate solution into a 15 cc. conical centrifuge tube, slowly add 2 cc. of serum and mix by inversion. Centrifuge for 10 minutes.

2. Measure 4 cc. of the clear supernatant into another conical centrifuge tube, add 1 cc. of glacial acetic acid and 9 cc. of the benzidine solution.

3. Cap the tube, place in ice water for 30 minutes, then centrifuge for 15 minutes at 3000 r.p.m.

4. Decant and discard the supernatant and drain the tube over filter paper for 3 minutes.

5. Add 14 cc. of acetone, suspend the precipitate in the acetone by mixing with a pointed fine glass rod, washing the rod with a little acetone when removing from the solution.

6. Cap the tube and centrifuge again for 15 minutes. Discard the supernatant and drain 5 minutes.

7. Add 1 cc. of the borate solution and dissolve by stirring. Place the tube in water at 60° C. to aid in the solution if necessary.

8. Add 10 cc. of water to the solution.

9. Into 2 test tubes pipet 2 cc. and 5 cc. of the dilute benzidine hydrochloride standard and add 8 cc. and 5 cc. of water respectively, followed by 1 cc. of the borate solution to each tube.

10. To each of the 3 tubes add 1 cc. of the naphthoquinone solution, mix and allow to stand 5 minutes.

11. Add 2 cc. of acetone to each tube, mix, and compare the unknown in the colorimeter with the standard nearer to it in color.

**Calculations.**—Set the standard at 20, then  $\frac{40}{\text{reading of unknown}} = \text{mg. of sulfur}$  per 100 cc. of serum if the 2 cc. standard is used or  $\frac{100}{\text{reading of unknown}}$  if the 5 cc. standard is used. If the result in terms of sulfate is desired, multiply the values obtained above by 3.

**Notes.**—1. Reagents should, of course, be free of sulfur. If glassware has been cleaned in dichromate cleaning fluid, it is essential that it be washed thoroughly with large quantities of water to be sure that all traces of sulfuric acid are removed.

2. Inorganic sulfur in normal sera varies from 0.9 to 1.6 mg. per 100 cc. or 2.7 to 4.8 when calculated as sulfate. It is increased in advanced glomerular nephritis and the uropathies due to obstruction, and polycystic kidney disease.

## CLARK AND COLLIP METHOD FOR DETERMINATION OF CALCIUM

**Principle.**—In this method (*Jour. Biol. Chem.*, 63: 461, 1925) the calcium precipitate as oxalate is determined by titrating the oxalic acid, liberated by acid, with standard permanganate solution.

**Apparatus.**—Tubes, centrifuge, 15 cc. Pyrex.

Buret, micro, graduated in 0.02 cc. intervals.

**Reagents.**—1. *Ammonium oxalate*.—Dissolve 4 gm. ammonium oxalate in water and dilute to 100 cc.



2. *Sulphuric acid*, approximately N/1:—Dilute 28 cc. of concentrated sulphuric acid to 1 liter.

3. *Potassium permanganate*, N/100:—Dilute 10 cc. of exact N/10 potassium permanganate (see page 785) to 100 cc. This solution is not sufficiently accurate for use and also changes its strength on standing. It should be titrated each day before a determination of calcium is made. Place 2 cc. of N/100 sodium oxalate solution in a centrifuge tube. Heat it in boiling water for 1 minute and titrate to the first pink color, persisting for about 15 seconds. Use the factor  $F = \frac{2}{\text{cc. permanganate}}$  in the calculation.

4. *Sodium oxalate*, N/100:—To a 100 cc. vol. flask add exactly 10 cc. of accurately standardized N/10 sodium oxalate (see page 785). Dilute to the mark with normal sulphuric acid. This solution is permanent and may be kept for standardizing the N/100 permanganate.

5. *Ammonium hydroxide*:—Dilute 2 cc. of ammonium hydroxide to 100 cc.

**Procedure.**—1. Collect about 10 cc. of blood in a dry syringe and transfer to a plain test tube; allow it to clot; then separate serum by centrifugation.

2. If sufficient serum has been obtained run a duplicate determination.

3. Pipet 2 cc. of water into a centrifuge tube, then 2 cc. of serum and 1 cc. of ammonium oxalate solution.

4. Mix thoroughly with a stirring rod.

5. Allow to stand 30 minutes.

6. Centrifuge at a high speed approximately 5 minutes. The precipitate must be well packed in the bottom of the tube.

7. Decant the supernatant liquid with care, then place the tube in a rack for 5 minutes to drain with the mouth of the tube resting on a piece of filter paper.

8. Wipe the mouth of the tube dry with a piece of filter paper.

9. With a pipet wash the sides of the tube and precipitate with 3 cc. of the dilute ammonium hydroxide solution.

10. Centrifuge and drain as before.

11. Add 2 cc. of the normal sulphuric acid, breaking the mat with the stirring rod.

12. Place the tube with the stirring rod in a boiling water bath for 1 minute.

13. Immediately titrate the solution with N/100 potassium permanganate solution. The end point is the faintest pink color that persists for about 1 minute.

**Calculation.**—T. cc. of permanganate used in titrating serum.

X = mg. of calcium per 100 cc. of blood serum.

F = Factor value of the permanganate solution.

$X = 10 \times F \times T.$

**Notes.**—1. The normal range is about 9 to 11 milligrams in 100 cc. of serum. In children and infants it is slightly higher. In tetany and after parathyroidectomy, there is a decrease. Low calcium is frequently found in acute rickets, pneumonia, and in some cases of epilepsy.

2. The centrifuge tubes should be perfectly clean. They should be kept in potassium dichromate sulphuric acid cleaning solution.

3. All glassware used in the determination must be rinsed with distilled water.

4. In titrating, the permanganate should be added very slowly at the beginning, as it takes a little time for the reaction to start and oxygen will be lost if permanganic

acid accumulates. The second drop should not be added until the pink color given by the first drop has disappeared. The titration temperature is important and should be 70° to 75° C. at the start and not lower than 60° C. at the end. Otherwise too much permanganate will be used. The centrifuge tube may be conveniently held in the water bath with a test-tube holder and may be stirred by giving it a gentle whipping motion. The end-point is to be taken as the faintest persisting pink color that can be recognized when looking down the tube against a white background; at this point no pink color is recognized if one looks through the tube.

### LOONEY AND DYER METHOD FOR DETERMINATION OF POTASSIUM

**Principle.**—In this method (*Jour. Lab. & Clin. Med.*, 28: 354, 1942) serum potassium is precipitated as the silver cobalti-nitrite compound from a protein-free filtrate from which chlorides have been removed. The potassium is estimated by comparing the color produced by coupling the precipitate with sulfanilamide and N-1, naphthylethylenediamine with a potassium standard similarly treated.

**Reagents.**—1. *Sodium tungstate solution*, 1.5 per cent.

2. *Copper sulfate solution*, 2.5 per cent.

3. *Silver nitrite solution*, 2.5 per cent and 40 per cent.

4. *Sodium hydroxide solution*, N/5 (approx.).

5. *Sodium cobaltinitrite solution*: (a) Dissolve 25 gm. of cobalt nitrate crystals in 50 cc. of water and add 12.5 cc. of glacial acetic acid. (b) Dissolve 120 gm. of sodium nitrite in 180 cc. of water. Add 210 cc. of B to all of A and aerate the solution under the hood until all the nitrous oxide fumes are driven off. Stored in the refrigerator, it keeps about 6 weeks. Filter each time before using.

6. *Silver-cobaltinitrite solution*: To 20 cc. of filtered cobaltinitrite solution, add 1 cc. of 40 per cent silver nitrate. Shake well and filter to remove traces of undissolved precipitate.

7. *Sulfanilamide solution*: 0.5 gm. of sulfanilamide in 100 cc. of 30 per cent acetic acid. Prepare fresh weekly.

8. *Coupler solution*: 0.1 gm. of N-1, naphthylethylenediamine dihydrochloride (Eastman or La Motte) in 100 cc. of 30 per cent acetic acid. Prepare fresh weekly.

9. *Washing mixture*: Mix 2 volumes of alcohol, 1 volume of ether and 2 volumes of water.

10. *Potassium Standard*.—Dissolve 223 mg. of potassium sulfate in water and dilute to the mark in a 1-liter volumetric flask. Dilute 10 cc. of this stock solution to the mark in a 100 cc. volumetric flask (3 cc. = 0.03 mg. potassium).

**Procedure.**—1. Into a clean test tube, pipet 0.5 cc. of serum, 7.0 cc. of distilled water, 1.0 cc. of 1.5 per cent sodium tungstate and 1.0 cc. of 2.5 per cent copper sulfate. Stopper, shake well and add 0.5 cc. of 2.5 per cent silver nitrate. Shake again, let stand 15 minutes and filter.

2. Pipet 3 cc. of the clear filtrate into a 15 cc. graduated centrifuge tube and 3 cc. of the diluted standard into a similar centrifuge tube.

3. To each add 1 cc. of 95 per cent alcohol, 1 cc. of distilled water and place both tubes in a water bath at 18° to 22° C. for 5 minutes.

4. Add to each tube 2 cc. of silver cobaltinitrite reagent, cap the tubes, mix the contents by swirling and replace them in the water bath for 2 hours.

5. Centrifuge for 15 minutes at about 2800 r.p.m.
6. With a capillary pipet remove the supernatant to the 0.2 cc. mark.
7. Wash with 7 cc. of the wash reagent, rinsing down the sides but disturbing the precipitate as little as possible.
8. Centrifuge for 15 minutes, decant, invert and drain over filter paper.
9. Repeat this washing twice more.
10. Add 10 cc. of 0.2N sodium hydroxide to each tube, mix by sidewise shaking and place in boiling water for 10 minutes.
11. Cool, make up to 10 cc. with water, stopper the tubes and mix thoroughly, remove the stoppers, cap the tubes and then centrifuge.
12. Pipet 2 cc. portions of the clear supernatant fluids into 100 cc. volumetric flasks and add to each 5 cc. of distilled water, 1 cc. of 50 per cent hydrochloric acid and 2 cc. of 0.5 per cent sulfanilamide.
13. Mix, let stand for 3 minutes and add 1 cc. of 0.1 per cent N-1, naphthyl-ethylenediamine dihydrochloride.
14. Dilute to the marks with water and read in the colorimeter after 5 minutes.

**Calculations.**—If the visual colorimeter is used, set the standard at 20, then

$$\frac{400}{\text{reading of unknown}} = \text{mg. of potassium per 100 cc. of serum.}$$

If the photoelectric colorimeter is used, use color filter No. 52 and set the zero with a reagent blank consisting of the contents of the standard flask except that water is used in place of the standard potassium solution, then

$$\frac{20}{\text{reading of standard}} (= \text{factor})$$

× reading of unknown = mg. of potassium per 100 cc. of serum.

**Notes.**—1. The factor is constant for the same set of reagents; the color reaction follows Beer's law up to about the equivalent of 30 mg. per cent of potassium.

2. Blood corpuscles contain about 4 times as much potassium as the serum, so that it is essential that the serum used be free from the slightest trace of hemolysis.

3. The potassium content of normal human serum varies between 16 and 22 mg. per cent; increased values have been obtained in pneumonia and other acute infections, Addison's disease, uremia and acute intestinal obstruction; decreased values have been found in hyperpituitarism.

### WEINBACH METHOD FOR DETERMINATION OF SODIUM

**Principle.**—In this method (*Jour. Biol. Chem.*, 110: 95, 1935) the sodium is precipitated as the triple salt of uranyl zinc sodium acetate from the deproteinized serum solution which is then titrated with standard sodium hydroxide solution, using phenolphthalein as indicator.

**Reagents.**—1. *Trichloroacetic acid solution*, 20 per cent.

2. *Uranyl zinc acetate solution*: (a) To 77 gm. of uranyl acetate add about 400 cc. of water and 14 cc. of glacial acetic acid. Dissolve with the aid of gentle heat while stirring, cool and dilute to 500 cc. in a volumetric flask. (b) To 231 gm. of zinc acetate add about 400 cc. of water and 7 cc. of glacial acetic acid. Dissolve with the aid of gentle heat and stirring, cool and dilute to 500 cc. in a volumetric flask. Heat both solutions and mix in a 2-liter Erlenmeyer flask while hot. Allow the mixed solutions to

stand 24 hours, then filter. Store in the dark. If a film forms on the surface, filter a small portion as needed.

3. *Acetone wash solution*: Prepare a small amount of the triple salt by adding 15 cc. of the uranyl zinc acetate reagent to 1 cc. of a 5 per cent solution of sodium chloride followed by the addition of 5 cc. of 95 per cent alcohol in small portions. Filter with suction and wash the precipitate four times with 5 cc. portions of alcohol followed by 4 washings with 5 cc. portions of ether, sucking dry after each addition of alcohol or ether. Add the precipitate to 1 liter of acetone, shake, let the covered flask stand overnight and remove excess precipitate from the acetone by filtration.

4. *Sodium hydroxide solution*, N/50.

5. *Sodium Standard*.—Dissolve exactly 1.0 gm. of sodium chloride in water and dilute to the mark in a 1-liter volumetric flask (1 cc. = 0.393 mg. of sodium and is equivalent to 6.84 cc. of N/50 sodium hydroxide).

**Procedure**.—1. In a 25 cc. Erlenmeyer flask mix 1 cc. of serum or plasma with 3 cc. of water. Add with shaking 1 cc. of 20 per cent trichloroacetic acid, mix well, let stand 10 minutes and filter.

2. Pipet 0.5 cc. of the clear filtrate into a 15 cc. centrifuge tube and add 5 cc. of the uranyl zinc acetate solution.

3. Add 0.3 cc. of 95 per cent alcohol and let stand for 5 minutes.

4. Again add 0.3 cc. of alcohol and let stand for a few minutes.

5. Repeat the addition of alcohol in small portions without greatly disturbing the precipitate until 2.1 cc. of alcohol have been added, the entire process of precipitation taking about 30 minutes.

6. Centrifuge, decant the supernatant and drain for a few minutes over a pad of filter paper.

7. Wash the precipitate once by blowing in 10 cc. of the acetone wash solution; centrifuge, decant, drain over a pad of filter paper and wipe the mouth of the tube.

8. Add 5 cc. of water to the precipitate in the centrifuge tube, mix by slight shaking and, when dissolved, transfer quantitatively to a 100 cc. Erlenmeyer flask, rinsing the centrifuge tube with 3 portions of water.

9. Add approximately 50 cc. of water and 0.5 cc. of 1 per cent alcoholic phenolphthalein solution.

10. Add N/50 sodium hydroxide from a microburet, graduated at 0.02 cc. intervals, to a just barely perceptible pink.

11. Titrate a distilled water blank using the same volume as used in the unknown in order to determine the amount of N/50 sodium hydroxide required to give the same end point as the unknown.

**Calculation**.—Subtract the blank titration from that of the unknown. The difference  $\times 57.5 =$  mg. of sodium per 100 cc.

**Notes**.—1. As a check on the procedure, determine the sodium in 0.5 cc. of the standard sodium solution, treating the solution exactly as the unknown filtrate.

2. The final volumes in the titrating flasks should be nearly alike since the end point is somewhat obscured due to the precipitate which forms during the titration; for this reason, the standard solution should be run concurrently with the unknown until sufficient practice is attained in determining the end point.

3. Normal serum or plasma contains 310 to 340 mg. of sodium per 100 cc. Because of the relatively large quantity of sodium present, it plays an important part in

the acid base balance of the blood. Increased values have been found in Cushing's disease, decreased values in Addison's disease, excessive sweating, and diabetes mellitis with acidosis.

### METHOD FOR DETERMINING THE ICTERUS INDEX

**Principle.**—This method is based upon a physical measurement of the yellow color of blood serum by colorimetric comparison with a standard 1:10,000 potassium dichromate solution, which arbitrarily represents unity in color density.

**Standard Solution.**—Dissolve 1 gram of potassium dichromate in 90 cc. of distilled water. Add 0.1 cc. of concentrated sulphuric acid and dilute to 100 cc. in a volumetric flask with water.

For use dilute 10 cc. to 1000 cc. with distilled water, making a 1:10,000 solution of potassium dichromate. The yellow color density of this solution represents one icterus index unit.

**Procedure.**—1. With a dry needle and syringe draw about 5 cc. of blood from a vein; place the blood in a plain tube, allow clot to form, centrifuge, and remove the serum. It is essential that the serum be free of the slightest visible hemolysis and chyle (blood should be taken after period of fasting).

2. Dilute 1 cc. of serum with a measured amount of physiological saline solution until its color approximates that of the standard.

3. Compare the serum in the Duboscq colorimeter with the icterus index standard solution set usually at 15 mm. When intense jaundice is present, the standard may be set at 30 mm.

4. The computation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of serum}} \times \text{dilution} = \text{Units of icterus index.}$$

5. As comparison is sometimes difficult in a colorimeter because of variations in shades and degrees of opalescence, the following standards may be employed:

Dissolve 1 gram potassium dichromate in water, add 2 drops concentrated sulphuric acid, and dilute to 100 cc. in a volumetric flask. Into 11 dry, clean, ordinary test tubes pipet 10, 5, 3, 2, 1.5, 1.2, 1, 0.7, 0.5, 0.3 and 0.1 cc. of the above dichromate solution, and with a 10 cc. Mohr pipet dilute the last 10 tubes to 10 cc. with distilled water. Mix. Clean 11 small test tubes (10x100 millimeters) with cleaning solution, wash and dry. Fill about  $\frac{2}{3}$  full with above solution. Label the tubes respectively 100, 50, 30, 20, 15, 12, 10, 7, 5, 3, 1. These may be tightly stoppered or preferably closed by sealing the glass. These labeled numbers correspond to icteric index units.

6. The normal icterus index varies from 4 to 6 units.

### METHOD FOR DETERMINING THE DIRECT VAN DEN BERGH REACTION

**Principle.**—This method is based upon the observation that bilirubinate reacts with the diazonium bodies of Ehrlich's diazo reagent in an aqueous medium without alcohol to form a reddish dye, azobilirubin.

**Ehrlich's Diazo Reagent.**—This is freshly prepared by mixing the A and B solutions in the proportion of 5 cc. of A with 0.15 cc. of B.

SOLUTION A

Sulphanilic acid .....	1 gm.
Hydrochloric acid, C.P.....	15 cc.
Distilled water, to .....	1000 cc.

SOLUTION B

Sodium nitrite, C.P. ....	0.5 gm.
Distilled water .....	100.0 cc.

Solution A will keep indefinitely. Solution B should be freshly prepared every 6 weeks.

**Procedure.**—1. Place 1 cc. of serum in a graduated centrifuge tube.

2. Slant the tube to an almost horizontal plane and allow 0.5 cc. of Ehrlich's diazo reagent to run down the wall of the tube from a pipet so that it will be overlaid on the serum.

3. The contact zone between the serum and the reagent is examined against a good background (sky or glazed glass window) for the development of a reddish ring, which, however faint, is indicative of the positive reaction.

4. If, in very weak reactions, the presence of a ring is suspected, it will become well defined in 1 or 2 minutes if the reaction is positive.

5. If the reddish ring is demonstrable within 2 minutes the reaction should be recorded as positive. If not demonstrable within 2 minutes but apparent within 10 minutes, the reaction should be recorded as "positive in — minutes."

**Notes.**—The direct van den Bergh reaction is normally negative. The use of this technic practically eliminates the former "delayed" reaction. Most positive reactions develop within 1 minute. The reaction is negative if no change occurs within 10 minutes.

## METHOD FOR DETERMINING THE INDIRECT VAN DEN BERGH REACTION

**Principle.**—In this method the colored azobilirubin is compared with an artificial color standard corresponding to a definite amount of bilirubin.

**Standard Bilirubin Solution.**—Dissolve 2.16 grams of anhydrous cobalt sulfate in distilled water and dilute to 100 cc. or dissolve 3.92 grams of hydrated cobaltous sulfate crystals in water, add 0.5 cc. sulfuric acid, C.P., and dilute to 100 cc. Either of these standards will keep indefinitely in the dark, and each is the equivalent of 0.5 milligrams of bilirubin per 100 cc., in the form of azobilirubin.

**Procedure.**—1. After the determination of the direct van den Bergh reaction, as described above, shake the tube containing the serum and diazo reagent and add about 3 cc. of 95 per cent alcohol (containing a trace of ether).

2. If a definite pinkish color fails to appear within 2 or 3 minutes, and only a white turbidity occurs, the reaction may be recorded as negative.

3. If a very definite pink or ruby color develops, add 1 cc. of saturated ammonium sulfate solution, shake gently, cap the tube, and centrifuge for 5 to 10 minutes.

4. On removal of the tube from the centrifuge, 3 layers will be seen: a bottom layer of clear colorless ammonium sulfate, a middle layer of compact precipitated

serum proteins, and an upper layer of pink or ruby red alcoholic solution of azobilirubin. Determine from the graduations on the centrifuge tube the volume in cubic centimeters of the upper colored layer, and calculate the dilution factor as follows:

$$\frac{\text{Volume of upper layer}}{\text{Volume of serum originally used}} = \text{dilution factor}$$

5. In the Duboscq colorimeter compare a portion of the upper layer (pipet or pour off into colorimeter cup) with the bilirubin standard solution. For weak reactions the standard may be set at 20 mm., for strong reactions, at 5 or 10 mm. The calculation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{dilution factor}}{2} = \frac{\text{milligrams of bilirubin}}{\text{per 100 cc. of serum}}$$

*Notes.*—1. Normally the indirect van den Bergh reaction shows 0.1 to 0.25 mg. bilirubin per 100 cc. serum.

2. When bilirubinate has been shown to be present, the middle layer of proteins will often be faintly pink in color, indicating a loss of pigment from the upper layer. This loss is not clinically relevant, but if one wishes to avoid it, van den Bergh has recommended that the serum first be diluted with distilled water before the addition of the diazo reagent, in order to break up the adsorption of bilirubinate by serum proteins. This modification of the technic will increase materially the yield of bilirubin in some sera, but will not do so in any serum unless the direct van den Bergh reaction has been found to be positive.

### MALLOY AND EVELYN METHOD FOR DETERMINATION OF BILIRUBIN

**Principle.**—In this method (*Jour. Biol. Chem.*, 119: 481, 1937) for bilirubin in serum or plasma, diazotized sulfanilic acid is coupled with bilirubin in methyl alcohol to produce a pink dye, the color of which is measured in the photoelectric colorimeter using green filter No. 54.

**Reagents.**—*Methyl alcohol, absolute, C.P.*

**Diazo blank solution:** Dilute 15 cc. of concentrated hydrochloric acid to 1 liter with distilled water.

**Solution A.**—Place 1.0 gm. of sulfanilic acid in a 1-liter volumetric flask; add 15 cc. of concentrated hydrochloric acid and dilute to the mark with distilled water. This solution keeps indefinitely.

**Solution B.**—0.5 per cent solution of sodium nitrite. Prepare fresh daily.

**Diazo reagent.**—Shortly before use, add 3 cc. of solution B to 100 cc. of solution A and mix.

**Standard Stock Bilirubin Solution.**—Place 40 mg. of pure bilirubin in a 100 cc. volumetric flask, add pure dry chloroform to dissolve and dilute to the mark with chloroform (1 cc. = 0.4 mg.). Store in the refrigerator in a brown bottle with a well fitted stopper. The solution keeps indefinitely.

**Dilute Standard Bilirubin Solution.**—Transfer 1 cc. of the stock solution to a 100 cc. volumetric flask and dilute to the mark with methyl alcohol (5 cc. = 0.02 mg.).

**Procedure.**—1. Dilute 1.0 cc. of serum or plasma to 10 cc. with distilled water.

2. Add to 3 colorimeter tubes marked B (blank), U (unknown) and S (standard) the following: Tube B, 5.0 cc. of methyl alcohol and 1.0 cc. of the diazo blank solution.

Tube U, 5.0 cc. of absolute methyl alcohol and 1.0 cc. of the diazo reagent.

Tube S, 5.0 cc. of the diluted bilirubin standard solution and 1.0 cc. of the diazo reagent.

3. To tubes B and U, add 4 cc. portions of the diluted serum or plasma, and 4 cc. of distilled water to tube S.

4. Mix by gentle inversion, treating all tubes uniformly, allow to stand 30 minutes and read in the colorimeter against a distilled water zero.

**Calculation.**—Subtract the reading of the blank from the reading of the unknown only. Then,  $\frac{5.0}{\text{reading of standard}}$  (= factor)  $\times$  corrected reading of unknown = bilirubin in mg. per 100 cc.

**Notes.**—1. The factor is constant and reproducible. Once determined in duplicate or triplicate, the standard need not be run simultaneously with each unknown.

2. Earlier conceptions of “free” and “combined” bilirubin are probably incorrect; apparently all bilirubin is bound to albumin.

3. Methyl alcohol releases all the bound bilirubin and also appears to catalyze the reaction with the diazo reagent.

4. In this method normal bilirubin values range between 0.2 to 0.4 mg. per 100 cc., although some blood samples are encountered which show practically none.

### BLOOR, PELKAN AND ALLEN METHOD FOR DETERMINATION OF CHOLESTEROL (MODIFIED)

**Principle.**—In this method for total cholesterol and cholesterol esters in serum or plasma (*Jour. Biol. Chem.* 52: 191, 1922) the cholesterol is extracted by an alcohol-ether mixture and the cholesterol determined colorimetrically in the filtrate before and after precipitation by digitonin.

**Reagents.**—1. *Chloroform, U.S.P.*

2. *Acetic anhydride-sulfuric acid mixture.*—In a dry, glass-stoppered cylinder mix 20 cc. of acetic anhydride with 2 cc. of concentrated sulfuric acid. Chill in the refrigerator. This mixture should be used within 1 hour of its preparation.

3. *Alcohol-ether.*—Mix 75 cc. of 95 per cent alcohol with 25 cc. of ether.

4. *Digitonin Solution.*—0.5 per cent solution in 95 per cent alcohol.

5. *Petroleum ether, B.P.*—35 to 60° C.

6. *Cholesterol Stock Standard.*—Dissolve exactly 160 mg. of cholesterol in about 50 cc. of chloroform. Transfer to a 100 cc. volumetric flask and dilute to the mark with chloroform (10 cc. = 16.0 mg.).

7. *Cholesterol Working Standard.*—Five cc. of stock standard is diluted to 100 cc. with chloroform (10 cc. = 0.8 mg.). Both standards keep well if evaporation is prevented by using well fitting ground glass-stoppered flasks and storing at a low temperature.

**Procedure.**—*Preparation of Extract.*—Place about 20 cc. of alcohol-ether mixture in a 25 cc. glass-stoppered volumetric flask. Add, slowly and with rotation of the flask, exactly 1.0 cc. of serum or plasma. The resulting precipitate should be finely



divided and free of clumps. Immerse the flask in hot water (*avoid open flame*) until the contents just come to a boil. Cool to room temperature under running tap water and dilute to the mark with the alcohol-ether mixture. Stopper the flask, shake vigorously and filter through a fluted filter paper (fat free, Whatman #40), covering the funnel with a watch glass to retard evaporation.

**Total Cholesterol.**—1. Pipet 10 cc. of filtrate into a 50 cc. beaker and evaporate just to dryness on a hot water bath (*do not use an open flame*).

2. Add 3 cc. of chloroform, stir with a fine glass rod, bring to boiling momentarily, allow the sediment to settle and carefully decant the clear liquid into a dry 10 cc. cylinder.

3. Repeat the chloroform extraction twice more with 3 cc. portions, adding the clear extracts to the same cylinder.

4. Allow to cool to room temperature and make up to 10 cc. with chloroform.

5. Place 10 cc. of cholesterol working standard in another 10 cc. cylinder.

6. Add to each cylinder, 2 cc. of the acetic anhydride-sulfuric acid mixture.

7. Stopper both cylinders, mix by side to side shaking, and place in a 30° C. water bath for 15 minutes.

8. Cool to room temperature for 5 minutes and compare in a colorimeter.

9. If the standard is set at 20 then  $4000 \div \text{reading of unknown} = \text{total cholesterol in mg. per cent of original sample}$ .

Or, if the unknown is set at 20, then  $10 \times \text{reading of standard}$  gives the same result.

**Cholesterol Esters.**—1. Pipet 10 cc. of the alcohol-ether filtrate into a 50 cc. beaker, add 1 cc. of digitonin solution, mix by rotation, and evaporate to dryness on a hot water bath (*do not use an open flame*).

2. Bake on water bath for 1 to 2 minutes.

3. Add about 10 cc. of petroleum ether. Immerse the beaker in hot water long enough for the contents to come to a momentary boil and decant the clear liquid into another 50 cc. beaker.

4. Repeat the extraction 3 more times.

5. Evaporate the combined petroleum ether extracts on the hot water bath (*avoid open flame*).

6. Extract the residue 3 times with 3 cc. portions of chloroform as for total cholesterol.

7. Develop and compare the colors in this chloroform extract and standard exactly as for total cholesterol.

8. The calculation is the same as for total cholesterol.

$$\frac{\text{mg. ester cholesterol}}{\text{mg. total cholesterol}} \times 100 = \text{per cent cholesterol ester.}$$

**Photoelectric Examination.**—Use filter No. 66. Determine the color values after setting the zero with a "blank" containing 5 cc. of chloroform plus 1 cc. of acetic anhydride sulfuric acid mixture.

The calculation is  $\frac{200}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{total or ester cholesterol in mg. per cent of original sample}$ .

**Notes.**—1. Normal total cholesterol is 150 to 250 mg. per 100 cc. of serum. Normal ester cholesterol is 60 to 75 per cent of total cholesterol.

2. Old and deteriorated material will lead to poor colors. Also all beakers, pipets, etc., must be perfectly dry.

3. Cholesterol is partly endogenous and partly exogenous; the latter is particularly derived from eggs, butter, meats and some vegetables.

4. Cholesterol has been found increased in diabetes mellitus with lipemia, in nephritis, complete obstruction of the common bile duct, and in some cases of cholelithiasis and arteriosclerosis. Decreased concentrations have been observed in pernicious anemia, cachexia of malignancy and in some cases of high fever.

### MICRO-KJELDAHL METHOD FOR DETERMINATION OF TOTAL PROTEIN

**Principle.**—From the ammonium nitrogen obtained by wet digestion of the serum, followed by nesslerization, is subtracted the nonprotein nitrogen of the same serum and the result multiplied by a factor to give total protein.

**Reagents.**—Same as for Nonprotein Nitrogen in Blood.

**Standard Ammonium Sulfate.**—Same as for Nonprotein Nitrogen in Blood.

**Procedure.**—1. One cc. of serum is diluted to the mark in a 50 cc. volumetric flask with physiological saline solution.

2. One cc. of this mixture is pipetted into a Folin digestion tube and the acid digestion with subsequent nesslerization carried out exactly as for Nonprotein Nitrogen in Blood.

3. If the unknown is set at 15, then  $50 \times$  reading of standard = mg. per cent of total nitrogen.

4. Carry out a nonprotein nitrogen determination on a separate portion of the serum as described on page 804.

**Calculation.**— $(\text{Total nitrogen} - \text{nonprotein nitrogen}) \times \frac{6.25}{1000} = \text{per cent total serum proteins.}$

### MICRO-KJELDAHL METHOD FOR DETERMINATION OF ALBUMIN AND GLOBULIN

**Principle.**—The globulin is precipitated from the serum and the albumin determined in the filtrate. The globulin is determined by difference.

**Reagents.**—1. *Nessler's solution*: see page 797.

2. *Standard ammonium sulfate*: see page 804.

3. *Sodium sulfate 23 per cent.*—Dissolve 23 gm. of the anhydrous salt by warming to about 50° C. Cool to 38° C. and make up to 100 cc. Keep in incubator to prevent crystallization.

4. *Ether, U.S.P.*

**Procedure.**—1. Determine the total protein of the serum by the micro-Kjeldahl method as described above.

2. Place 7.5 cc. of the 23 per cent sodium sulfate solution in a 20 cc. test tube. Add 0.5 cc. of the serum and mix thoroughly. Add about 3 cc. of ether, stopper and shake vigorously for a few seconds. Carefully remove the stopper, cap the test tube and centrifuge for 10 minutes. By this means the globulin is collected cleanly in the ether layer above the albumin solution.

3. Pass a thin 1 cc. pipet between the ether layer and the wall of the test tube so that the tip enters the albumin solution. Withdraw 1 cc. of the albumin solution, wipe off the outside of the pipet and transfer to a Folin digestion tube.

4. Digest and determine the nitrogen exactly as for nonprotein nitrogen.

5. Determine the nonprotein nitrogen of the serum.

**Calculations.**—*Albumin.*—Set the nesslerized unknown at 15 when  $(16 \times \text{reading of standard} - \text{N.P.N.}) \times \frac{6.25}{1000} = \text{per cent of albumin in serum.}$

*Globulin.*—Per cent of total protein minus per cent of albumin = per cent of globulin.

*Notes.*—1. If a blood urea N. has been determined at the same time and is normal, it is not necessary to do a nonprotein nitrogen. A subtraction of 25 mg. may be used for the nonprotein nitrogen in the calculation.

2. Normal protein content of serum is 6 to 7.5 per cent.

Normal albumin content of serum is 4.0 to 5.0 per cent.

Normal globulin content of serum is 2.0 to 2.5 per cent.

3. Albumin-globulin ratio: 1.5 to 2.5:1.

4. In nephrosis the total protein content is decreased, as well as the albumin but the globulin is increased. Globulin is also increased in various anaphylactic conditions, in malignancy and in toxic conditions of scarlet fever, erysipelas, pneumonia and in toxemia of pregnancy.

### GREENBERG METHOD FOR DETERMINATION OF ALBUMIN AND GLOBULIN

**Principle.**—In this method (*Jour. Biol. Chem.* 82: 545, 1929) globulin is precipitated from blood serum by sodium sulfate solution. Albumin is determined colorimetrically in the filtrate by means of a phenol reagent. Total protein is determined by the same reagent. Total protein minus albumin equals globulin.

**Reagents.**—1. *Standard Tyrosine Solution.*—Weigh exactly 0.2 grams of tyrosine, transfer to a liter volumetric flask, dissolve in and dilute to the graduation using N/10 hydrochloric acid solution.

2. *N/10 Hydrochloric Acid Solution.*—For preparation see page 784.

3. *10 per cent Sodium Hydroxide Solution.*—For preparation see page 784.

4. *22.2 Per Cent Sodium Sulphate Solution.*—Weigh 22.2 grams of the sodium sulphate and introduce into a 100 cc. volumetric flask. Add hot water to dissolve and dilute to the graduation.

5. *Phenol Reagent.*—Introduce into a 2-liter Pyrex Erlenmeyer flask 100 grams of sodium tungstate, 25 grams of sodium molybdate, 700 cc. of water, 50 cc. of phosphoric acid and 100 cc. of concentrated hydrochloric acid. Reflux gently for 10 hours, then add 150 grams of lithium sulphate, 50 cc. of water and a few drops of bromine. Remove the condenser and boil this mixture 15 minutes to remove excess bromine. Bromine fumes are poisonous, therefore the mixture should be boiled in a fume closet. Cool, dilute to 1 liter and filter. The solution should have no greenish tint. Preserve in a glass stoppered brown bottle.

6. *0.85 Per Cent Sodium Chloride Solution.*—In a 100 cc. volumetric flask dissolve 0.85 gram of sodium chloride in water and dilute to the graduation.

**Procedure.**—1. To prepare the albumin solution, place 9.5 cc. of the sodium sulfate solution in a 20 cc. test tube. Add 0.5 cc. of serum and mix. Add about 3 cc. of ether, stopper the tube and shake vigorously for a few seconds. Remove the stopper, cap the test tube and centrifuge 10 minutes. Pipet 5 cc. of the lower albumin sulfate solution into a 50 cc. volumetric flask.

2. Dilute 1 cc. of serum with 9 cc. of the 0.85 per cent sodium chloride solution and mix. Pipet 2 cc. into another 50 cc. volumetric flask.

3. Pipet 5 cc. of the tyrosine standard solution into a third 50 cc. volumetric flask.

4. To each of the 3 volumetric flasks add about 35 cc. of water, exactly 4 cc. of 10 per cent sodium hydroxide solution and 3 cc. of the phenol reagent. Dilute to the marks with water and mix.

5. After 5 minutes compare the albumin and total protein solutions against the tyrosine solution in the colorimeter.

**Calculation.**—The tyrosine factor for total protein is 16.0 and for albumin 16.6. If the unknowns are set at 10 then:

Per cent total protein =  $0.8 \times$  reading of the standard.

Per cent albumin =  $0.664 \times$  reading of the standard.

Per cent globulin = per cent total protein — per cent of albumin.

### LOONEY AND WALSH METHOD FOR DETERMINATION OF ALBUMIN AND GLOBULIN

**Principle.**—In this turbidometric method (*Jour. Biol. Chem.* 130: 635, 1939) the turbidities caused by total protein and globulin precipitants are measured directly in the photoelectric colorimeter. Albumin is calculated by difference.

**Reagents.**—*Gum ghatti*, 2 per cent.

*Sulfosalicylic acid*, 5 per cent. Prepared on the indicated basis from a sulfosalicylic acid which does not give a pink color in solution.

*Sodium chloride solution*, 0.85 per cent.

*Ammonium sulfate, saturated solution.*

**Procedure.**—1. Determine the total protein and globulin content of a clear specimen of human serum by the micro-Kjeldahl method.

**Globulin Factor.**—2. Dilute 1 cc. of this standard serum with 9 cc. of the sodium chloride solution. Place 1 cc. of this dilute serum in a colorimeter tube and add 2 cc. of the gum ghatti solution followed by 3 cc. of the saturated ammonium sulfate solution. Mix by inversion several times. Read in the colorimeter after 10 minutes against a distilled water zero using a No. 42 filter.

The globulin factor =  $\frac{\text{per cent globulin content of standard.}}{\text{reading of standard}}$

3. **Total Protein Factor.**—Dilute 1 cc. of the standard serum with the saline solution to 100 cc. in a volumetric flask, or dilute 1 cc. of the diluted serum used for the globulin standardization to 10 cc. with saline solution. Place 2 cc. of this dilution in a colorimeter tube, add 0.5 cc. of the gum ghatti solution and 2.5 cc. of the 5 per

cent sulfosalicylic acid. Mix by inversion several times and read in the colorimeter against a distilled water zero using a No. 42 filter.

The total protein factor =  $\frac{\text{per cent protein content of standard}}{\text{reading of standard}}$

These factors should be determined in duplicate or triplicate. Once determined, they are valid as long as the reagents remain unchanged and need not be determined when the unknown is run.

4. To determine *globulin*: Prepare a 10 per cent dilution of the unknown serum and treat exactly as for the determination of the globulin factor.

5. To determine *total protein*: Prepare a 1 per cent dilution of the unknown serum and treat exactly as for the determination of the total protein factor.

**Calculations.**—1. Per cent total protein = reading of the unknown  $\times$  the total protein factor.

2. Per cent globulin = reading of unknown globulin  $\times$  globulin factor.

3. Per cent albumin = total protein — globulin.

**Notes.**—1. After the protein precipitants are added, the contents of the tubes should be mixed by inversion only. Vigorous shaking will cause excessive entrainment of minute air bubbles which will affect the colorimeter readings.

2. The 10-minute waiting period is usually sufficient for the small number of air bubbles to disappear. However, a longer waiting period may be used since the gum ghatti maintains a uniform suspension for more than 30 minutes.

### CAMPBELL AND HANNA METHOD FOR DETERMINATION OF FIBRINOGEN

**Principle.**—In this method (*Jour. Biol. Chem.* 119: 15, 1937) the fibrinogen, precipitated by sulfite, is determined by micro-Kjeldahl followed by nesslerization.

**Reagents.**—1. *Sodium Sulfite*, 12.5 per cent: from the anhydrous salt.

2. *Sulfuric acid*, 50 per cent; see page 782.

3. *Nessler's solution*; see page 797.

**Procedure.**—1. In a Folin digestion tube graduated at 50 cc., place 0.5 cc. of plasma; add 9.5 cc. of the sulfite solution, mix and allow to stand at 37° C. for 10 minutes.

2. Centrifuge 5 minutes, decant and drain the supernatant fluid.

3. Wash the precipitate with 5 cc. of the sulfite solution, breaking up the precipitate with a fine glass rod.

4. Centrifuge, decant and drain.

5. Add 1 cc. of 50 per cent sulfuric acid and proceed with the nitrogen determination by micro-Kjeldahl method as given on page 798.

**Calculation:** With the nesslerized unknown set at 15, then  $2 \times$  reading of standard = mg. per cent of fibrinogen nitrogen. To convert to per cent fibrinogen multiply by  $\frac{6.25}{1000}$ .

### MYLONE, WINTERNITZ AND DE SÜTÖ-NAGY METHOD FOR DETERMINATION OF FIBRINOGEN

**Principle.**—In this method (*Jour. Biol. Chem.* 143: 21, 1942) the fibrinogen in oxalated plasma is precipitated by protamine and the nitrogen of the precipitate determined by the micro-Kjeldahl procedure.

**Reagents.**—1. *Protamine Sulfate Solution*, 1 per cent in saline.

2. *Calcium Acetate Solution*, 1.58 per cent.

3. *Trichloroacetic Acid Solution*, 10 per cent in absolute alcohol.

**Procedure.**—1. In a Folin digestion tube graduated at 50 cc., place 25 cc. of saline and add 1.0 cc. of plasma.

2. Add 1 cc. of the protamine solution and mix.

3. After 1 minute add 3 cc. of the calcium acetate solution and place the tube in the refrigerator for 1 hour.

4. Centrifuge 20 minutes, pour off the supernatant, add 30 cc. of cold distilled water and centrifuge for 10 minutes. Discard the wash water.

5. Add 30 cc. of the trichloroacetic acid solution and loosen the mat of fibrinogen with the end of a glass rod. Again centrifuge, after rinsing off the glass rod with a little of the trichloroacetic acid solution and discard the supernatant.

6. Determine the nitrogen content according to the method given for total nitrogen by micro-Kjeldahl on page 798.

**Calculation.**—If the nesslerized unknown is set at 15, then  $1 \times$  reading of standard = mg. per cent of fibrinogen nitrogen. To convert to per cent of fibrinogen multiply by  $\frac{6.25}{1000}$ .

**Note.**—This method is not affected by the type of anticoagulant, and the results are somewhat higher than by the older methods.

### COPPER SULFATE METHOD FOR DETERMINATION OF SPECIFIC GRAVITIES OF WHOLE BLOOD AND PLASMA

This method, described by Phillips, Van Slyke, Dole, Emerson, Hamilton, Archibald, Stanley and Plazin,\* makes it possible with 3 or 4 drops of blood, a medicine dropper, and small bottles of copper sulfate solution to determine the specific gravity of the blood, and from it the hemoglobin content within 10 per cent. By examining in a like manner the serum or plasma from the same blood one can determine also the plasma protein concentration and increase the accuracy of the hemoglobin estimation to  $\pm 2$  per cent.

The results can be used as follows:

1. To assist in ascertaining the results of hemorrhage.

2. To estimate decrease in plasma volume indicated by hemoglobin increase, and to decide whether the plasma volume decrease is due to loss of water (dehydration of cholera, dysentery, exposure), or to loss also of plasma proteins (extravasation in burns, trauma, etc.).

\* From the United States Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research.

3. To assist in deciding whether blood replacement therapy requires administration of saline solution or plasma or whole blood.

4. To follow the results of such therapy and decide whether it has been adequate, and when it must be repeated.

5. If the number of cases exceeds the amount of blood and plasma available, the method will assist in deciding which cases must receive it and which may be able to do without.

6. Besides these acute conditions, the method will assist in diagnosing the different types of anemia and in detecting various pathological conditions, partially summarized in a later section, in which the plasma proteins become diluted or concentrated.

**Principle.**—The technic consists of letting drops of plasma or whole blood fall into a graded series of solutions of copper sulfate of known specific gravity, and noting whether the drops rise or fall in the solutions. Each drop, on entering the solution, becomes encased in a sack of copper-proteinate, and remains as a discrete drop without change of gravity for 15 or 20 seconds, during which its rise or fall reveals its gravity relative to that of the solution. The size of the drops does not have to be constant, hence no special pipet is needed for delivering the drops. No temperature correction is needed, because the temperature coefficient of expansion of the copper sulfate solutions approximates that of blood and plasma. This method is capable of measuring gravities to  $\pm 0.00005$ , which is more than ten times the accuracy required. The copper sulfate solution automatically cleans itself after each test, because within a minute or two after the test is completed the material of the drop settles to the bottom as a precipitate. The standard  $\text{CuSO}_4$  solutions are prepared by dilution of a saturated solution, hence a balance is not needed to weigh the  $\text{CuSO}_4$ .

For accurate work, viz., gravities within  $\pm 0.0002$ , a series of copper sulfate solutions graded at intervals of 0.001 in specific gravity is used; 20 solutions cover the plasma range 1.015 to 1.035 and 40 cover the whole blood range, 1.035 to 1.075. For rougher work with gravities accurate to  $\pm 0.001$ , 16 solutions at intervals of 0.004 suffice to cover the entire range of blood and plasma.

**Reagents.**—*Saturated copper sulfate solution:* Four pounds of "fine crystals," or pulverized, copper sulfate are placed in a 4-liter bottle. About 2500 cc. of distilled water is added and the bottle is stoppered and shaken vigorously for a total of 5 minutes, which need not be continuous. (Three minutes has been found sufficient, even at  $0^\circ \text{C}$ ., to saturate this solution if the sulfate is well pulverized.) As soon as the shaking is finished the temperature of the solution is taken to the nearest half degree Centigrade and is recorded. (It will be a little cooler than the water was before the saturation, because the saturation process absorbs heat.) After taking the temperature the solution is *immediately* decanted off the crystals and is filtered, to remove fine suspended crystals, through cotton or dry filter paper into a clean dry 4-liter bottle. The solution is at once used to make up a stock solution of gravity 1.100. (It is preferable not to let the saturated solution stand long before using, as if it cools some of the copper sulfate may crystallize and change the concentration.) The undissolved sulfate can be used again.

*Stock copper sulfate solution of gravity 1.100:* The volume of saturated solution indicated in Table 53 is measured in a 500 cc. graduated cylinder and poured into a 1-liter volumetric flask. The upturned cylinder is allowed to drain into the flask for 30 seconds. The flask is then filled to the mark with water and is inverted several

times to mix the solution. The mixing results in a contraction, so that the meniscus now falls below the mark. The flask is let stand for a minute until the solution drains down from the neck. Then enough additional water is added to bring the volume to 1 liter, the solution is mixed, and then poured into a clean, dry, 4-liter bottle. The same 1-liter volumetric flask is used to prepare 3 more liters of the stock copper sulfate solution of gravity 1.100. Each time before the flask is used again it is rinsed with water and the rinsings are discarded.

The saturated solution, the stock solution and the standard solutions next described must be prepared at within  $5^{\circ}$  C. of the same temperature. The coefficients of expansion of the saturated and stock copper sulfate solutions are slightly but definitely greater than that of water, so that if, for example, the saturated solution and stock solution were prepared at  $35^{\circ}$  C., and the standard solutions at  $20^{\circ}$  C., the standards would have more copper sulfate than intended, enough to increase the gravity by about 0.001.

Once prepared, the standard solutions may be used at any temperature within  $\pm 15^{\circ}$  or  $20^{\circ}$  of the temperature at which they were made up.

The accuracy of the stock solution of gravity 1.100 can be checked by weighing 100 cc. in a volumetric flask, and then weighing 100 cc. of distilled water at the same temperature in the same flask. The copper sulfate solution should weigh 1.1000 times the weight of the water. This check is desirable because the accuracy of the method depends on the accuracy of the stock solution.

*Preparation of standard solutions in 100 cc. portions:* The standard solutions are prepared in 100 cc. portions when 4-ounce bottles are available for storage.

For the standard of 1.075 gravity, 74 cc. of stock solution of gravity 1.100 are measured from a buret into a 100 cc. volumetric flask, the flask is filled to the mark with water, and the solution is mixed and transferred to a labeled 4-ounce bottle, which is stoppered to prevent evaporation.

To prepare the standard of gravity 1.074, the 100 cc. flask is rinsed once with water and the buret is refilled from a 250 cc. Erlenmeyer flask containing the stock solution. Then 73 cc. of the stock solution are measured into the volumetric flask and diluted to 100 cc.

The same procedure is carried through for preparation of the entire series down to 1.015, which covers the extreme ranges for blood and plasma. If gravities on ascitic fluid and transudates are desired, the series is extended to 1.008. For each standard the number of cc. of stock solution *less by 1* than the number indicated in the second and third decimal places of the desired gravity is measured into the rinsed 100 cc. flask and diluted to the mark.

If there were no contraction when the stock solution is mixed with water one would dilute 75 cc. of the stock to 100 cc. to get a gravity of 1.075, etc. However, there is a contraction which is empirically corrected for by taking 1 cc. less of the stock. It happens conveniently that the same 1-cc. correction serves for the entire range, 1.075 to 1.008, over which its use yields gravities correct within  $\pm 0.0003$ .

**Procedure.**—1. Tourniquets should not be applied for more than 1 minute. Longer application may force so much fluid out of the blood that the concentration is measurably increased. In conditions of shock capillary blood may contain 30 to 40 per cent more cells than venous blood; hence in such conditions capillary blood cannot serve as a sample of circulating blood.



2. *Whole blood* may be delivered directly from the syringe and needle in which the blood was drawn into the copper sulfate solutions. If, however, the blood is transferred to a test tube containing anticoagulant, *the cells and plasma must be thoroughly mixed immediately before a sample is drawn* into a medicine dropper for the gravity test. For this purpose, the tube containing the blood is (a) inverted 10 times, or (b) a glass rod with a mushroom end is raised and lowered through the blood 10 times, just before the sample is drawn into the dropper. Gross error in hemoglobin estimation could result if the blood sample were taken from blood in which partial settling of the cells had occurred.

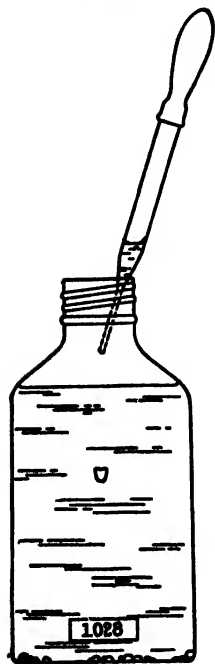


FIG. 324.—BOTTLE FOR DETERMINATION OF SPECIFIC GRAVITY OF BLOOD OR PLASMA BY COPPER SULFATE METHOD

(From Phillips, Van Slyke, Dole, Emerson, Jr., Hamilton and Archibald, *Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma*, by permission of the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research.)

*Plasma* gravity is best determined on plasma from blood which is treated with heparin, 0.2 mg. per cc. blood, as anticoagulant, since the heparin exerts no measurable effect on the gravity results. Almost equally good is Heller and Paul's mixture of 3 parts ammonium oxalate and 2 parts potassium oxalate, *if the amount used does not exceed 1 mg. per cc. of blood*. Sodium citrate cannot be used as anticoagulant. In effective concentrations it exerts too great an effect on the gravities.

3. The drop of serum, plasma or whole blood is delivered from a height of about 1 cm. above the solution from a medicine dropper, or from a syringe needle. It is preferable to use small drops for the reason that they permit more tests before the standard solution must be changed. Therefore, a medicine dropper with a fine tip is preferable to a coarse one. Greasing the sides of the tip with vaselin also reduces the size of the drop, especially if the vaselin is mixed with a little caprylic alcohol. When the drop is delivered it is convenient to steady the dropper on the edge of the bottle (Fig. 324).

The delivered drop breaks through the surface film of the solution and penetrates 2 to 3 cm. below the surface; within 5 seconds the momentum of the fall is lost and the drop then either begins to rise, or becomes stationary, or continues to fall. The gravity of the drop relative to the solution does not change appreciably until the drop has been immersed in the solution for another 10 or 15 seconds, and there is ample time to note its behavior during this interval. If the drop is lighter than the test solution it will rise, perhaps only a few millimeters and may begin to sink immediately afterwards. If the drop is of the same gravity as the standard test solution it will become stationary for this interval and then fall. If the drop is heavier it will continue to fall during the interval. *In summary, the behavior during the 10 seconds after the drop has lost the*

*momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution; if it rises at all during this period it is known to be lighter than the standard.*

4. For general work it may suffice to determine the gravities to  $\pm 0.001$ . For this only 16 standard solutions with gravity intervals of 0.004 covering the range from

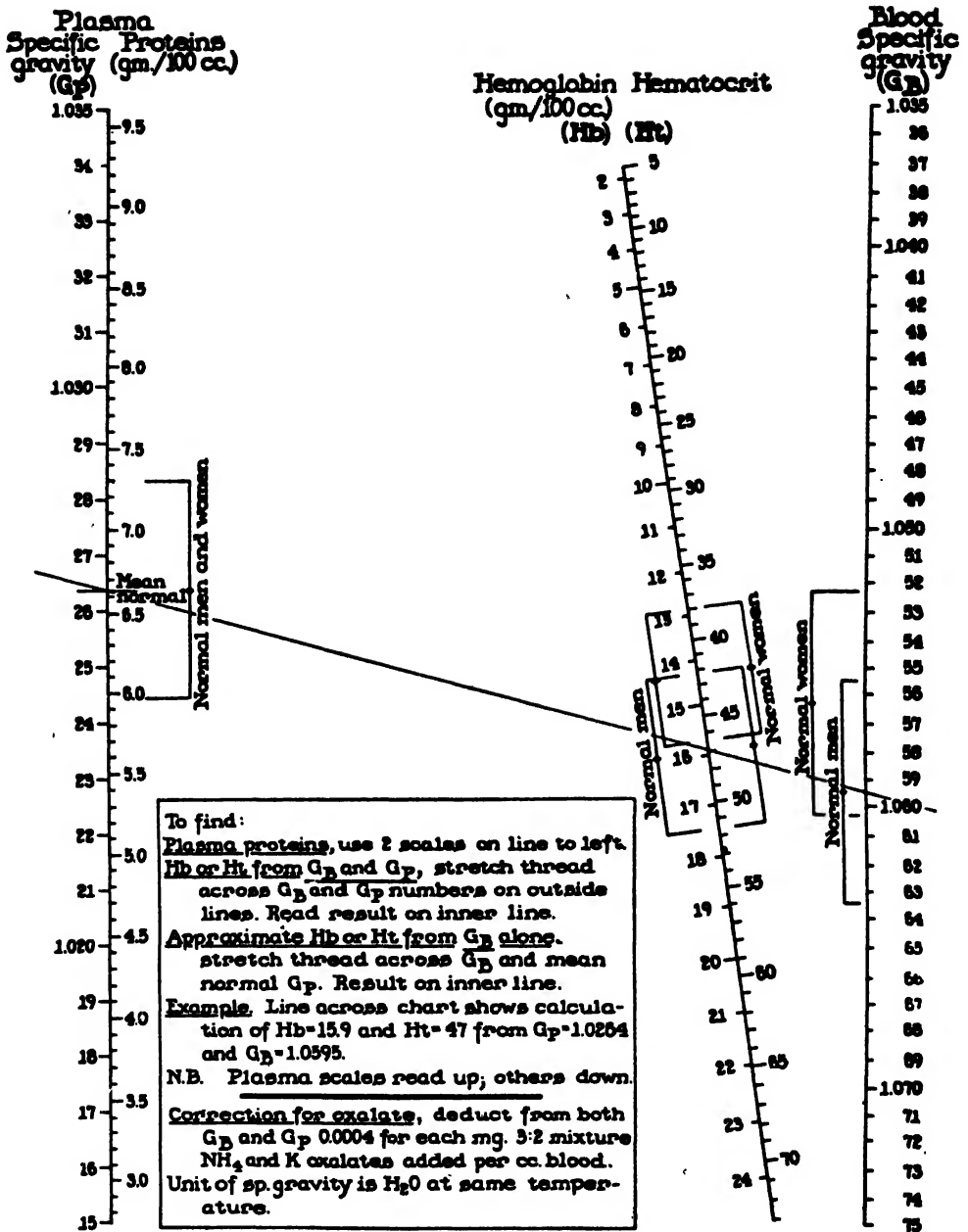


FIG. 325.—LINE CHART FOR CALCULATING PLASMA PROTEINS, HEMOGLOBIN AND HEMATOCRIT FROM GRAVITIES OF PLASMA AND BLOOD

(From Phillips, Van Slyke, Dole, Emerson, Jr., Hamilton and Archibald, *Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma*, by permission of the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research.)

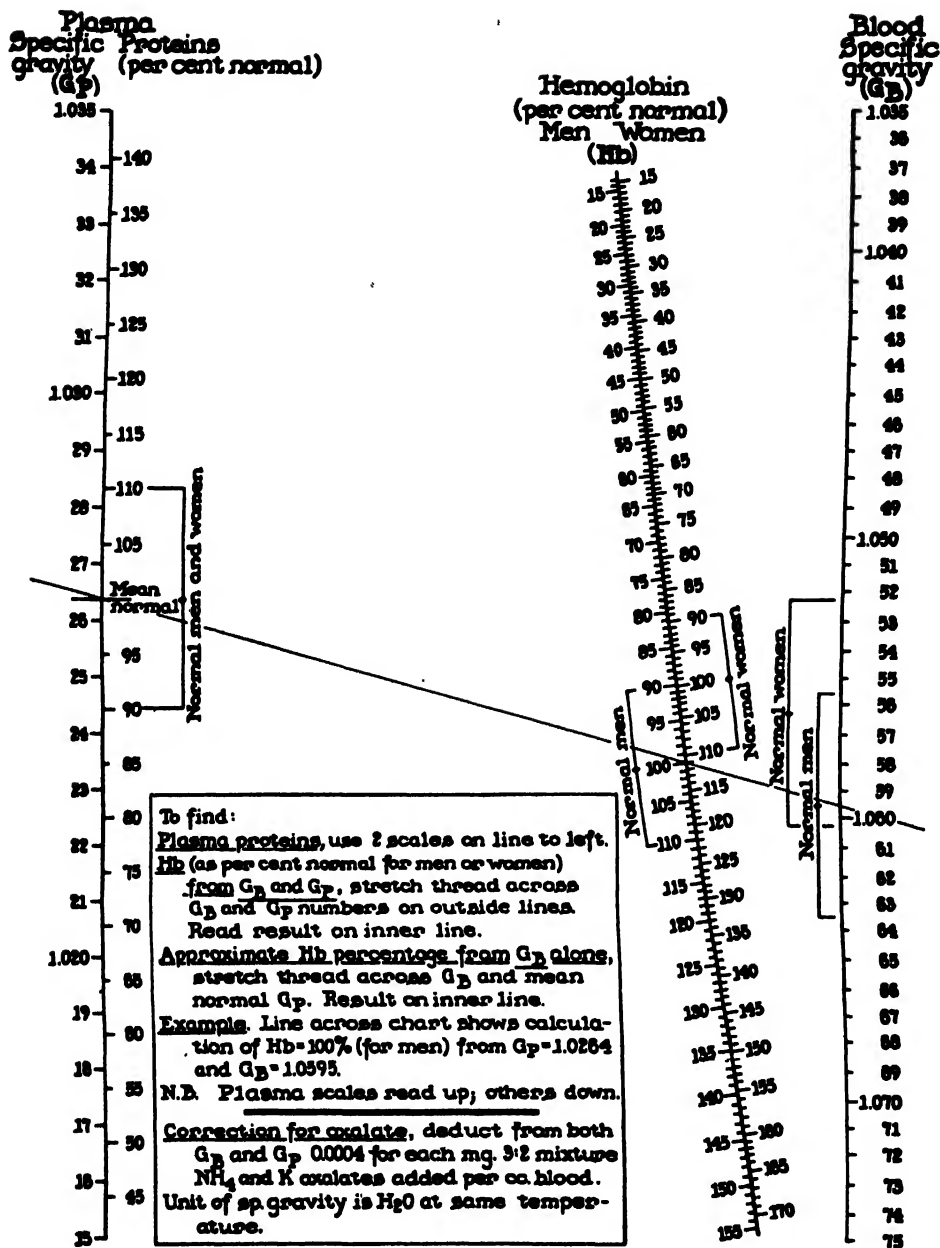


FIG. 326.—LINE CHART FOR CALCULATING PERCENTAGES OF NORMAL PLASMA PROTEINS AND HEMOGLOBIN FROM GRAVITIES OF PLASMA AND BLOOD

(From Phillips, Van Slyke, Dole, Emerson, Jr., Hamilton and Archibald, *Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma*, by permission of the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research.)

**CC. OF SATURATED COPPER SULFATE SOLUTION TO BE DILUTED TO 1 LITER TO GIVE THE STOCK SOLUTION OF SPECIFIC GRAVITY 1.100 \***

Temperature in °C. or °F. refers to the temperature of the saturated solution at the time of saturation (end of shaking for 5 minutes).

Temperature °C.	°F.	cc.	Temperature °C.	°F.	cc.	Temperature °C.	°F.	cc.
10.0	50.0	578	20.0	68.0	488	30.0	86.0	425
10.5	50.9	573	20.5	68.9	484	30.5	86.9	423
11.0	51.8	568	21.0	69.8	480	31.0	87.8	420
11.5	52.7	563	21.5	70.7	477	31.5	88.7	417
12.0	53.6	558	22.0	71.6	473	32.0	89.6	414
12.5	54.5	553	22.5	72.5	469	32.5	90.5	412
13.0	55.4	548	23.0	73.4	466	33.0	91.4	409
13.5	56.3	543	23.5	74.3	463	33.5	92.3	406
14.0	57.2	539	24.0	75.2	460	34.0	93.2	403
14.5	58.1	534	24.5	76.1	456	34.5	94.1	401
15.0	59.0	529	25.0	77.0	453	35.0	95.0	398
15.5	59.9	525	25.5	77.9	450	35.5	95.9	395
16.0	60.8	521	26.0	78.8	447	36.0	96.8	392
16.5	61.7	516	26.5	79.7	445	36.5	97.7	390
17.0	62.6	512	27.0	80.6	442	37.0	98.6	387
17.5	63.5	508	27.5	81.5	439	37.5	99.5	384
18.0	64.4	504	28.0	82.4	436	38.0	100.4	381
18.5	65.3	500	28.5	83.3	434	38.5	101.3	379
19.0	66.2	496	29.0	84.2	431	39.0	102.2	376
19.5	67.1	492	29.5	85.1	428	39.5	103.1	373
20.0	68.0	488	30.0	86.0	425	40.0	104.0	370

\* From Phillips, Van Slyke, Dole, Emerson, Jr., Hamilton and Archibald, *Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma*, by permission of the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research.

1.016 to 1.076 will be needed. An error of 0.001 in plasma gravity affects plasma proteins by 0.3 grams per 100 cc.; additive errors of 0.001 in the gravities of both plasma and whole blood affect hemoglobin results by 5 per cent.

**Calculations.**—Line charts for the conversion of plasma and whole blood gravities to plasma protein concentrations, hemoglobin concentrations and hematocrit percentages have been prepared by standard methods, and are given in Figures 325 and 326. The calculations are made by laying a straight edge or stretched thread as directed on the charts. The brackets on the scales indicate normal ranges.

**Corrections to Observed Gravities for the Effects of Addition of Oxalates or Removal of Fibrinogen.**—Addition of oxalates raises the gravity of whole blood and of plasma; on the other hand, removal of fibrinogen by clotting when no anticoagulant is used yields serum, which has a gravity lower than that of plasma. These effects are so small that for clinical studies the errors introduced may ordinarily be neglected, and figures for plasma proteins and hemoglobin may be calculated with sufficient accuracy by applying the observed gravity values of blood and plasma or serum directly to the line charts. However, if more than 1 mg. of oxalate mixture per cc. of blood is used, or if the greatest precision is desired, corrections are applied as follows:

*Corrections for added anticoagulants.*—No corrections are needed if heparin, 0.1 or 0.2 mg. per cc. of blood, is used. For each mg. of the ammonium-potassium oxalate mixture added per cc. of blood subtract 0.0004 from the observed  $G_B$  and the observed  $G_P$ . If a tube with 5 mg. of oxalate receives the expected 5 cc. of blood, the correction to  $G_P$  and  $G_B$  is therefore 0.0004. Neglect of the 0.0004 correction would lead to an overestimation of the plasma proteins by 0.1 gm. per 100 cc. of plasma, and of hemoglobin by 0.1 gm. per 100 cc. of blood, errors which may usually be neglected. If, however, the volume of blood placed in the tube is less than 5 cc. the oxalate concentrations will be greater, and the corrections to  $G_B$  and  $G_P$  will be as follows: for 4 cc. of added blood — 0.0005; for 3 cc. — 0.0007; for 2 cc. — 0.0010; for 1 cc. — 0.0020.

**Total Plasma Protein Concentration From Specific Gravity Data.**—The formula and the normogram scale for calculating plasma proteins are based upon Moore's and Van Slyke's data, which were based chiefly on pathological plasma. For *normal* human plasma, it is now found that 376 is a more accurate factor than 343, and mean normal plasma proteins are slightly over 7.0 gms. per 100 cc.

#### VAN SLYKE METHOD FOR DETERMINATION OF OXYGEN CAPACITY

1. The Van Slyke blood gas apparatus (see Fig. 323) is washed out twice with water before each analysis in order to remove the alkali used to absorb carbon dioxide in any previous analysis.

2. The entire apparatus is filled with mercury, including the capillaries above the upper stop cock. For 2 cc. of blood, introduce 6 cc. of water, 0.3 cc. of 1 per cent saponin (Merck) solution and 2 or 3 drops of caprylic alcohol into the apparatus, and free of air by evacuation and 15 seconds of shaking. The extracted air is expelled and the extraction is repeated until no more air is obtained.

3. The air-free solution is now drawn down and trapped in the wide branch of the apparatus below the lower stopcock. The stopcock is turned and mercury run very slowly upwards through the apparatus in order to collect the film of water left on the inside and this film is expelled through the outlet capillary on the left side. Mercury is now run into the bottom of the cup and any moisture in the cup is dried by filter paper.

4. Two cc. of oxalated blood are now drawn into the 50 cc. chamber from a pipet by lowering the mercury reservoir, and are trapped near the bottom of the chamber. While the upper stopcock remains open, the apparatus is shaken for 2 or 3 minutes, thus saturating the blood with oxygen.

5. The mercury is run up again into the 50 cc. chamber, collecting the blood at the top. When the blood reaches the upper stopcock, this is closed. The lower stopcock is turned so that the previously trapped air-free water is allowed to rise in the chamber. The lower stopcock is closed and the apparatus shaken a few seconds to mix the water and blood. The blood is laked in  $1\frac{1}{2}$  minutes.

6. From 0.10 to 0.12 cc. of potassium ferricyanide solution (20 grams per 100 cc. water) is measured into the cup and introduced into the chamber with the laked blood. (The ferricyanide may be measured with sufficient accuracy as 3 drops from a dropper which delivers 1 cc. in 25 to 30 drops.) A mercury seal is made and the apparatus is evacuated and shaken for 2 to 3 minutes.

7. The evolved gas is composed of oxygen, nitrogen and carbon dioxide. In order to absorb the carbon dioxide the leveling bulb is placed at such a height that the mercury in it is slightly below the level of the mercury in the apparatus, and 0.5 cc. of N/2 sodium hydroxide solution (previously saturated with air by shaking) is admitted from the cup of the apparatus and allowed to trickle *slowly* down the inner wall of the chamber. If the latter part of the solution enters as a solid column instead of running down the walls, it is dislodged by letting a little mercury pass down through it in a fine stream. In any case it is usually necessary to dislodge with a drop of mercury the last drop of alkali solution which adheres just below the stopcock.

8. The fluid mixture is trapped in the bulb below the lower stopcock, mercury is run up through the left arm and the reading is made in the same manner as in the determination of carbon dioxide.

9. The oxygen capacity may also be determined with the manometric apparatus. For details see Van Slyke and Neill: *Jour. Biol. Chem.*, 61: 523, 1924.

**Calculation.**—The volume of gas measured ( $V$ ) is  $O_2 + N_2 + H_2O$  and correction must be made for temperature ( $t$ ) barometric pressure ( $B$ ), water vapor ( $W$ ) and physically dissolved air (2.1 cc. of air being dissolved in 100 cc. of blood at 20° C.).

$$V \left( 50 \times \frac{B - W}{760 (1 + 0.00367t)} \right) - 2.1 = \text{volume per cent oxygen capacity}$$

The value in parentheses is a factor which can be obtained from the table.

**TABLE FOR THE CALCULATION OF HEMOGLOBIN CONTENT**

Volumes per cent oxygen capacity  $\times 0.746$  = grams hemoglobin per 100 cc. blood. Volumes per cent oxygen capacity  $\times 4.78$  = per cent hemoglobin.\*

Temperature, Centigrade	Factor = $50 \times B - W / 760 (1 + 0.00367t)$
15	$46.6 \times B / 760$
16	$46.4 \times B / 760$
17	$46.2 \times B / 760$
18	$45.95 \times B / 760$
19	$45.75 \times B / 760$
20	$45.5 \times B / 760$
21	$45.3 \times B / 760$
22	$45.05 \times B / 760$
23	$44.85 \times B / 760$
24	$44.6 \times B / 760$
25	$44.4 \times B / 760$
26	$44.15 \times B / 760$
27	$43.9 \times B / 760$
28	$43.65 \times B / 760$
29	$43.4 \times B / 760$
30	$43.15 \times B / 760$

\* Based on Haden's average normal of 15.6 grams.

Blood can be kept in an ice box at 60° C. for at least 24 hours before any appreciable change takes place. After a certain time blood will absorb oxygen through the oil, and the values will increase. The opposite happens when the blood is kept in the laboratory where it cannot be expected to keep constant more than 2 hours. After that interval the oxygen content diminished rapidly, probably on account of bacterial action. (Lundsgaard, *J. Biol. Chem.*, 33: 143, 1918.)

**Oxygen Unsaturation.**—1. Volume per cent oxygen unsaturation equals volume per cent oxygen capacity minus volumes per cent oxygen bound by hemoglobin in venous blood.

2. This latter determination is made in the same manner as the oxygen content but the calculation is changed as follows:

$$V \left( 50 \times \frac{B - W}{760 (1 + 0.00367t)} \right) - 1.5 = \text{volumes per cent oxygen}$$

bound by hemoglobin in venous blood.

### WONG METHOD FOR DETERMINATION OF IRON

**Principle.**—In this method (*Jour. Biol. Chem.*, 77: 409, 1928) iron is separated from hemoglobin by means of sulfuric acid and potassium persulfate. The proteins are precipitated with tungstic acid and the iron in the filtrate is determined colorimetrically by the addition of potassium thiocyanate.

**Reagents.**—1. *Sodium Tungstate Solution.*—Transfer 10 grams of sodium tungstate to a 100 cc. volumetric flask, dissolve in water and dilute to the graduation.

2. *Potassium Persulphate Solution.*—Introduce into a glass-stoppered bottle approximately 7 grams of potassium persulphate. Add approximately 100 cc. of water. Shake well, then let stand. The excess salt will settle to the bottom. Use only the supernatant fluid.

3. *Potassium Thiocyanate Solution.*—Transfer 146 grams of potassium thiocyanate to a 500 cc. volumetric flask. Dissolve in water and dilute to the graduation. Pour into a glass-stoppered bottle and add 20 cc. of acetone to assist in preserving the solution.

4. *Iron Standard Solution.*—Dissolve exactly 0.7 gm. of ferrous ammonium sulfate in about 50 cc. of water and add 20 cc. of 10 per cent sulfuric acid. Warm slightly and add, with stirring, N/10 permanganate solution until a permanent pink color is obtained. Transfer to a 1-liter volumetric flask and dilute to the mark (1 cc. = 0.1 mg. iron).

**Procedure.**—1. Carefully pipet 0.5 cc. of oxalated blood into a 50 cc. volumetric flask.

2. Add 2 cc. of the concentrated sulphuric acid. Mix.
3. Add 2 cc. of the potassium persulphate solution. Mix.
4. Add approximately 25 cc. of water. Mix.
5. Add 2 cc. of the sodium tungstate solution. Mix.
6. Cool to room temperature and dilute to the graduation with water.
7. Mix thoroughly, then filter.
8. Pipet 20 cc. of the clear filtrate into a 25 cc. stoppered cylinder and label B.
9. Pipet 1 cc. of the iron standard solution into another 25 cc. stoppered cylinder and label S.
10. Add 0.8 cc. of the concentrated sulphuric acid to the standard cylinder. Dilute with water to the 20 cc. graduation. Cool to room temperature.
11. Add 1 cc. of the potassium persulphate solution to each cylinder.
12. Add 4 cc. of the potassium thiocyanate solution to each cylinder.
13. Mix and compare in the colorimeter.

**Calculation:**

$$\frac{S \times 50}{B} = \text{milligrams of iron per 100 cc. of blood.}$$

or set B at 10 mm. when  $S \times 5 =$  milligrams of iron per 100 cc. of blood.

$$\frac{S \times 20}{B} = \text{volumes per cent of oxygen capacity of blood.}$$

Milligrams of iron per 100 cc. of blood divided by 3.35 = grams of hemoglobin per 100 cc. of blood.

For photoelectric comparison the colors are developed as for visual comparison along with a blank containing 2.0 cc. of concentrated sulfuric acid and 2 cc. of saturated persulfate solution diluted to 50 cc.

Use color filter No. 54 and a distilled water zero setting. Subtract the blank reading from both the reading of the standard and unknown.

**Calculation:**

$$\frac{50}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{mg. iron per 100 cc. blood.}$$

or:

$$\frac{14.9}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{gm. of hemoglobin per 100 cc. blood.}$$

*Notes.*—1. If the color of B in the iron determination is too light for comparison, the cup may be set at 25 mm. when  $S \times 2 =$  mg. of iron per 100 cc. of blood.

2. All reagents except the standard must be iron-free. The blank determination as used in the photoelectric modification is an automatic check on this impurity in the reagents.

3. Both the blank and the factor are constant for any one set of reagents.

## KING AND ARMSTRONG METHOD FOR DETERMINATION OF SERUM PHOSPHATASE

**Principle.**—In this method (*Canad. Med. Assoc. Jour.*, 31: 376, 1934) the activity of phosphatase is measured in acid or alkaline substrates, containing disodium phenyl-phosphate, by its ability to liberate phenol, the amount of which is determined colorimetrically.

**Reagents.**—1. *Buffer Substrate at pH 5.*—Dissolve 21 gm. of citric acid in about 20 cc. of water and add 200 cc. of normal sodium hydroxide. Dissolve separately 1.09 gm. of disodium-monophenyl-phosphate in about 300 cc. of water. Mix the 2 solutions and dilute to 1000 cc. in a volumetric flask. Add 5 cc. of chloroform. Keep in refrigerator.

2. *Buffer Substrate at pH 9.3.*—Transfer to a 1-liter volumetric flask 1.09 gm. disodium-monophenyl-phosphate and 10.3 gm. sodium-diethyl barbiturate (barbital sodium). Dissolve and dilute to the mark. Add 5 cc. of chloroform. Keep in refrigerator.

3. *Sodium Carbonate, 20 per cent.*

4. *Phenol Reagent (Folin and Ciocalteu).*—In a 2-liter Florence flask place 100 gm. of sodium tungstate, 25 gm. of sodium molybdate and 700 cc. of water. When dissolved, add 50 cc. of 85 per cent orthophosphoric acid and 100 cc. of concentrated hydrochloric acid. Boil gently for 10 hours under a reflux condenser (cork stopper).



Cool, add 150 gm. of lithium sulfate, 50 cc. of water and a few drops of liquid bromine. Boil the mixture in a hood without the condenser for about 15 minutes to remove the excess bromine. Cool, pour into a liter cylinder and dilute to the mark. The finished solution should have no greenish tint. It should be kept in a bottle with a well fitting, ground glass stopper. For use in the tests 1 volume is diluted with 3 volumes of water.

5. *Stock Standard Phenol Solution*.—Dissolve 1 gm. of crystalline phenol in N/10 hydrochloric acid and make up to 1 liter with N/10 hydrochloric acid. Transfer 25 cc. of this solution to a 250 cc. ground glass-stoppered Erlenmeyer flask, add 50 cc. of N/10 sodium hydroxide solution and heat to 65° C. To the hot solution add 25 cc. of N/10 iodine solution, stopper the flask and let stand at room temperature for 30 to 40 minutes. Add 5 cc. of concentrated hydrochloric acid and titrate with N/10 thio-sulfate solution. Using the appropriate factors for the iodine and thiosulfate solutions which convert them to exact N/10 normal, the phenol in mg. per 100 cc. is  $6.27 \times (25 \text{ cc. N/10 iodine} - \text{cc. N/10 thiosulfate})$ . The solution keeps indefinitely in the cold.

6. *Standard Phenol Solution*.—This should contain exactly 10 mg. per 100 cc. Measure from a buret into a 100 cc. volumetric flask the quantity of stock standard phenol solution which is obtained by the fraction

$$\frac{1000}{\text{mg. phenol per 100 cc. of stock (item 5)}}$$

and dilute to the mark. Keeps at least 3 months in the refrigerator.

7. *Phenol Reagent*.—*Phenol Solution*.—This is the colorimeter standard and is made fresh before use by diluting 15 cc. of diluted phenol reagent and exactly 5 cc. of standard phenol (item 6) to 50 cc. in a volumetric flask.

**Procedure**.—Tests are run in duplicate. 1. In 4 test tubes place two 10 cc. portions each of pH 5 and pH 9.3 buffer substrate solutions.

2. Place tubes in a water bath at 37.5° C. and allow to remain long enough for the solutions to reach this temperature.

3. Add 0.5 cc. of serum to each tube, stopper with rubber stoppers, invert twice and allow to remain in the water bath for exactly 30 minutes.

4. Add 4.5 cc. of the diluted phenol reagent to each tube and mix by inverting several times.

5. In 2 other tubes place 10 cc. portions each of pH 5 and pH 9.3 buffer substrate solution. Add to each 4.5 cc. of diluted phenol reagent followed by 0.5 cc. of serum and mix (blank).

6. Centrifuge all tubes for 2 to 3 minutes.

7. Pipet 10 cc. of supernatant from each of the 6 tubes into 6 clean test tubes.

8. Pipet 10 cc. of the colorimeter standard phenol solution (item 7) into another test tube.

9. Add 2.5 cc. of the 20 per cent sodium carbonate to each of the 7 tubes and after 20 minutes compare in the colorimeter.

**Calculations**.—Since the diluted standard phenol solution contains 1.0 mg. per 10 cc. which is diluted 1:10 and 10 cc. of this solution used for comparison, if the unknown or blanks are set at 30, then  $\frac{\text{reading of standard}}{30} \times \frac{5}{50} \times 1 \times \frac{15}{10} \times \frac{100}{0.5} =$  the number of mg. of phenol per 100 cc. of serum. Or the phenol content of unknown or blank in mg. per 100 cc. = the reading of the standard.

The phosphatase activity, either acid or alkaline, in *King-Armstrong* units = reading of the standard against the unknown minus the reading of the blank against the unknown.

*Notes.*—1. If the phosphatase activity is high, giving very deep colors, the unknowns should be set at 15 and the calculation given above multiplied by 2.

2. If the phosphatase activity is over 100 units, hydrolysis products have probably interfered with the reaction and it is best to repeat the tests using 0.5 cc. of a 1:5 dilution of serum. The results are then multiplied by 5.

3. It is important that the time of incubation be strictly adhered to and the phenol reagent be added after exactly 30 minutes.

4. Acid phosphatase values under 5 King-Armstrong units are normal.

5. Alkaline phosphatase values under 13 King-Armstrong units are normal.

6. Values of acid phosphatase of over 5 are suspicious of prostatic carcinoma; over 10 are diagnostic for prostatic carcinoma with metastases.

7. Elevated alkaline phosphatase values are found in liver disease of the obstructive type, malignant bone tumors, Paget's disease, rickets and pregnancy.

### BODANSKY METHOD FOR DETERMINATION OF SERUM PHOSPHATASE

**Principle.**—In this method (*Jour. Biol. Chem.*, 101: 93, 1933) the activity of phosphatase is measured in acid or alkaline substrates, containing glycerophosphate, by its ability to liberate phosphorus, the amount of which is measured colorimetrically.

**Reagents.**—1. *Sodium Hydroxide*, N/10.

2. *Acetic Acid*, N/1: Transfer about 60 cc. of glacial acetic acid to a 1-liter volumetric flask and dilute to the mark with water. Titrate 5 cc. of this solution with N/10 sodium hydroxide using a few drops of 0.5 per cent alcoholic phenolphthalein solution as indicator:

$$\frac{50}{\text{cc. of 0.1N sodium hydroxide}} = \text{factor by which the volume of this acetic acid solution is multiplied to obtain the amount of this solution to use in order to be equivalent to the prescribed volumes of N/10.}$$

3. *Stock Glycerophosphate.*—Place 3 cc. of petroleum ether and about 80 cc. of water in a 100 cc. volumetric flask. Add 1.0 gm. of sodium betaglycerophosphate  $5\frac{1}{2}\text{H}_2\text{O}$  (Eastman) and 0.85 gm. of sodium diethyl barbiturate. Dissolve and bring the aqueous level to the mark. Mix and keep in the refrigerator.

4. *Alkaline Phosphate Substrate.*—Into a 100 cc. volumetric flask place 3 cc. of petroleum ether, 50 cc. of the stock glycerophosphate and 2.8 cc. of N/10 sodium hydroxide. Dilute the aqueous layer to the mark and mix. The pH should be  $10.8 \pm 0.1$ . Keep in refrigerator.

5. *Acid Phosphate Substrate.*—Into a 100 cc. volumetric flask place 3 cc. of petroleum ether, 50 cc. of stock glycerophosphate and 5 cc. of N/1 acetic acid. Dilute the aqueous layer to the mark and mix. The pH should be  $5.0 \pm 0.1$ . Keep in refrigerator.

6. *Trichloroacetic acid*, 40 per cent.

**Procedure.**—1. Place 9 cc. of the alkaline substrate into a test tube.

2. Place 9 cc. of the acid substrate into another test tube.

3. Place both tubes in a water bath at  $37.5^{\circ}\text{C}$ .
4. After the contents of the tubes have attained the water bath temperature, add to each 1 cc. of serum, stopper, mix and return to the water bath for 1 hour.
5. While the digestion is occurring, place 9 cc. of the alkaline substrate into a test tube and 9 cc. of the acid substrate into another test tube. These are the blank or control tubes. Add to each 2 cc. of the 40 per cent trichloroacetic acid and 1 cc. of serum. Stopper, shake and filter.
6. After exactly 1 hour, add 2 cc. of the trichloroacetic acid to the 2 tubes in the water bath. Stopper, shake and filter.
7. Place 6 cc. of the 4 filtrates into four 10 cc. graduated cylinders and proceed with the determination of phosphorous exactly as in the Fisk and Subbarow method described on page 820.

**Calculations.**—With the unknown set at 20, the phosphorous content per 100 cc. of serum is 0.4 times the standard reading. From the results of the heated tubes subtract the results of the respective unheated tubes. The remainders represent the phosphorus liberated due to phosphatase activity per 100 cc. (Bodansky units).

**Notes.**—1. Inhibition of hydrolysis usually occurs when values over 60 units have been obtained. In such cases repeat the determinations using 1 cc. of a 1:5 dilution of serum and multiply the results by 5.

2. The normal range for alkaline phosphatase is about 2 to 9 units; for acid phosphatase values in excess of 1.2 units are probably abnormal.

3. Abnormal values by this method have the same indications as for the preceding method.

## CHERRY AND CRANDALL METHOD FOR DETERMINATION OF LIPASE

**Principle.**—In this method (*Am. Jour. Physiol.*, 100: 266, 1932) the fatty acids which are liberated from olive oil by the lipolytic action of serum are determined by volumetric titration.

**Reagents.**—1. *Sodium hydroxide* N/20 made by diluting N/10 sodium hydroxide with an equal volume of water.

2. *Phosphate Buffer, pH 7.*—Transfer 2.27 gm. of potassium dihydrogen phosphate, anhydrous, to a 100 cc. volumetric flask. Dissolve and dilute to the mark with water. Transfer 2.97 gm. of disodium hydrogen phosphate, anhydrous, to a 100 cc. volumetric flask. Dissolve and dilute to the mark with water. Mix 3 cc. of the first solution with 10 cc. of the second solution. The pH should be 7 to 7.1.

3. *Olive oil emulsion, 50 per cent.*—This can be obtained from Wyeth and Brother, Philadelphia, or the Abbott Laboratories.

**Procedure.**—1. In one 10 cc. glass-stoppered cylinder measure 2 cc. of olive oil emulsion, 1 cc. of serum, 0.5 cc. of phosphate buffer and 3.5 cc. of water. In a second control cylinder place 2 cc. of olive oil emulsion, 0.5 cc. of phosphate buffer and 4.5 cc. of water.

2. Stopper the cylinders, shake thoroughly and incubate at  $40^{\circ}\text{C}$ . for 24 hours.

3. Transfer the contents of the cylinders to 50 cc. Erlenmeyer flasks, rinsing the cylinders with a small amount (about 3 cc.) of neutral alcohol. Add 3 drops of 0.5 per cent alcoholic phenolphthalein solution and titrate with the N/20 sodium hydroxide to a faint permanent pink.

**Calculation.**—Subtract the number of cc. of N/20 sodium hydroxide used for the control tube from that used in the serum tube. Results are expressed as cc. of N/20 required to neutralize the fatty acids liberated by 1 cc. of serum.

**Notes.**—1. Normal serum varies between 0.5 and 1.0 unit.

2. Values in excess of 1.5 cc. are frequently found in acute pancreatitis and in patients with carcinoma of the pancreas.

### SOMOGYI METHOD FOR DETERMINATION OF SERUM AMYLASE

**Principle.**—In this method (*Jour. Biol. Chem.*, 125: 399, 1938) the reducing sugar formed by the action of the amylase of serum on starch is determined by the Folin-Wu method.

**Reagents.**—1. *Sodium tungstate solution*, 10 per cent.

2. *Copper Sulfate solution*, 5 per cent.

3. *Starch solution.*—Place 50 gm. of pure rice starch or U.S.P. cornstarch in a liter glass-stoppered cylinder, add 500 cc. of approximately N/100 hydrochloric acid and shake intermittently during the course of an hour. After sedimentation, pour off the acid, add about 500 cc. of 0.05 per cent sodium chloride and shake thoroughly. After sedimentation, pour off the salt solution and repeat the washing with another 500 cc. portion of salt solution. Finally spread out the starch on a pad of filter paper and allow it to air dry.

Grind 15 gm. of the dried starch in a mortar with 50 cc. of water while 900 cc. of water are heated to a boiling in an Erlenmeyer flask. Transfer the ground starch paste to the boiling water with vigorous agitation, using 50 cc. of water to rinse the mortar. Heat on a boiling water bath for 30 minutes, keeping the mouth of the flask covered with a beaker. Cool, cover with toluene and store in the refrigerator. Keeps a few days.

4. *Sodium chloride solution.*—Dissolve 10 gm. of sodium chloride in water and transfer to a 1-liter volumetric flask. Add 3 cc. of N/10 hydrochloric acid and dilute to the mark with water.

**Procedure.**—1. In each of two 14 to 16 mm. test tubes place 5 cc. of the starch paste and 2 cc. of the sodium chloride solution; warm to incubator temperature (37° to 38° C.).

2. Add 1 cc. of serum to 1 tube and 1 cc. of water to the other tube (blank); stopper the tubes, mix and incubate for 35 minutes.

3. The amylase activity is then stopped in both tubes by the addition to each of 1 cc. of the copper sulfate solution and 1 cc. of the sodium tungstate solution. Mix and filter.

4. In a third test tube (control) place 5 cc. of water, 2 cc. of the sodium chloride solution, 1 cc. of serum and 1 cc. each of the sodium tungstate and copper sulfate solutions. Mix and filter.

5. Determine the reducing sugars on 2 cc. portions of each filtrate by the Folin-Wu method described on page 810.

**Calculation.**—The *amylolytic activity* is defined as the mg. of reducing sugars, expressed as glucose, per 100 cc. of serum; therefore, subtract the reducing sugar found in the blank and control from that of the unknown and the difference is the amylolytic value of the serum. Calculate as per the following example:

Plasma and starch gave a value of 350 mg. per 100 cc.

Starch blank alone gave a value of 25 mg. per 100 cc.

Serum blank alone gave a value of 108 mg. per 100 cc.

$350 - 25 - 108 = 217$  mg., the amylase concentration.

Normal values range from 40 to 110, averaging about 60. High values are generally indicative of acute pancreatitis. Low values are generally indicative of fibrosis and atrophy of the pancreas or hepatic disease. Normal or subnormal values are commonly observed in perforated peptic ulcers; moderately increased values are generally indicative of posterior perforated ulcers near or at the pancreas.

### BARKER METHOD FOR DETERMINATION OF THIOCYANATES

**Principle.**—In this method (*Jour. Am. Med. Assoc.*, 106: 762, 1936) the thiocyanate is determined by the measurement of the color produced by ferric salt on the deproteinized blood filtrate.

**Reagents.**—1. *Trichloroacetic Acid Solution*, 5 per cent.

2. *Ferric Nitrate Solution.*—Dissolve 5 gm. of crystallized ferric nitrate in about 50 cc. of water in a 100 cc. volumetric flask. Add 2.5 cc. of concentrated nitric acid and dilute to the mark with water.

3. *Standard Thiocyanate Solution.*—This is most conveniently prepared from the thiocyanate solution used in the blood chloride determination (1 cc. = 0.994 mg. of thiocyanate). For use dilute 2.01 cc. of this solution in a 100 cc. volumetric flask with 80 cc. of 5 per cent trichloroacetic acid and dilute to the mark with water (10 cc. = 0.2 mg. of thiocyanate).

**Procedure.**—1. Measure 16 cc. of the trichloroacetic acid into a 50 cc. Erlenmeyer flask. Add slowly and with constant shaking 4 cc. of plasma or serum. Stopper the flask and shake vigorously. Let stand 10 minutes and filter. The filtrate should be clear; if not, re-filter through the same filter paper.

2. Place 10 cc. of filtrate in a test tube.

3. Place 10 cc. of the dilute standard in another test tube.

4. Add to each tube 2 cc. of the ferric nitrate reagent, mix and read in the colorimeter within 5 minutes.

**Calculation.**—If the standard is set at 20, then  $\frac{20 \times .2}{\text{reading of unknown}} \times \frac{100}{2}$  or  $\frac{200}{\text{reading of unknown}} = \text{mg. per cent of thiocyanate in plasma or serum.}$

Or, if the unknown is set at 20, then  $\frac{1}{2}$  the reading of the standard = the unknown in mg. per cent.

**Notes.**—1. The colors fade on standing; colorimetric readings should not be delayed.

2. A maximum level of 10 mg. per 100 cc. is believed to be optimal in the treatment of hypertension. A content of more than 15 mg. per cent may produce toxic effects.



**Procedure For Sulfanilamide In Urine.**—1. Pipet 1 cc. of urine into a 10 cc. cylinder, add 1 cc. of 15 per cent trichloroacetic acid, dilute to the 10 cc. mark and filter if necessary.

2. Pipet 2 cc. of filtrate into a 50 cc. volumetric flask, add 2.5 cc. of 4N hydrochloric acid and dilute to the mark with water.

For *free sulfanilamide*, 10 cc. of the second dilution are treated exactly as was the 10 cc. of blood filtrate.

For *total sulfanilamide*. Ten cc. of the second dilution are heated for 1 hour without further addition of acid and treated as the blood filtrate.

**Calculations.**—1. If the unknown is set at 10, then the sulfanilamide content in mg. per cent is:

$$\begin{array}{rccccccc} 5.0 \times & \text{reading of standard, if 0.02 mg. standard was used.} \\ 12.5 \times & \text{" " " " 0.05 " " " "} \\ 25 \times & \text{" " " " 0.10 " " " "} \end{array}$$

**Notes.**—1. While it is somewhat more accurate to determine other sulfonamide derivatives by color comparison with standards prepared from the same compound administered, the increase in accuracy probably does not justify the maintenance of the large numbers of standards that would be involved. Further, it may not be possible to obtain the various compounds in sufficient purity for the preparation of standards. For clinical work the conversion factors may be employed if sulfanilamide is used as the standard. Obviously it is essential that the laboratory know which drug has been administered:

For sulfapyridine	multiply the sulfanilamide result by 1.45
For sulfathiazole	" " " " " 1.48
For sulfadiazine	" " " " " 1.47
For sulfamethylthiazole	" " " " " 1.54
For sulfaguanidine	" " " " " 1.25

The above factors are based on the anhydrous molecular weights, *i.e.*, the molecular weight of sulfanilamide is 172 and that of sulfapyridine is 249 and the conversion factor is  $\frac{249}{172} = 1.45$ . This procedure may be followed for such new sulfonamide compounds as they appear.

2. Colorimetric comparisons may be made in the photoelectric instrument using a 1:40 instead of a 1:20 blood dilution, color filter No. 54 and a distilled water zero. With well made reagents the blank reading is usually negligible. Use the standard containing 0.02 mg. per 10 cc. This will correspond to a concentration of 8 mg. per cent if a 1:40 dilution of blood is used. The factor then is  $\frac{8}{\text{reading of standard}}$ . This factor is then multiplied by the reading of the unknown to give mg. per cent of sulfanilamide in the blood.

3. Color proportionality in the photoelectric colorimeter is good over a wide range and only the one standard need to be used.

## METHOD FOR DETERMINATION OF BASAL METABOLIC RATE

**Principle.**—The rate of absorption of oxygen by the blood from the lungs under specified conditions is determined by either the open or the closed method.

1. *Open Method.*—The patient inspires ordinary outdoor air and all air that he expires during a known period of time is caught in a suitable receptacle and measured. A sample is removed and analyzed for oxygen, carbon dioxide, and nitrogen in the Haldane-Henderson gas analysis apparatus, and the oxygen absorption and carbon dioxide excretion of the patient is calculated. For this method, which requires training in gas analysis, see Peters and Van Slyke.\*

2. *Closed Method.*—The patient breathes into and out of a container of oxygen in which the carbon dioxide he produces is absorbed, either for a fixed length of time, during which the decrease in volume of oxygen is noted (Benedict-Roth and Sanborn apparatuses), or during the absorption of a fixed volume, one liter, of oxygen, for which the time required is noted (Jones apparatus). From the rate of absorption of oxygen, the rate of heat production per hour is determined, and compared with that of a normal individual of the same biometric measurements. The percentage variation from the latter is called the basal metabolic rate.

**Materials.**—*Apparatus.*—Closed circuit respiration apparatus, with mouthpiece (sterilized by boiling), nose clip, thermometer, kymograph with recording paper and ink. Bed, couch, stretcher, or other object upon which patient may recline comfortably. Watch. Clinical thermometer. Barometer calibrated in millimeters. Scales for weighing patient. Measuring device for obtaining his height.

*Chemicals.*—Soda lime, oxygen, pure, in compression cylinder.

**Procedure.**—If the patient is in the hospital he is given a light supper the night before the determination; is given no breakfast; is kept in bed; is taken to the metabolism station on a stretcher, and is given absolute rest for a  $\frac{1}{2}$  hour before the determination. An out-patient is told to eat a light supper at least 12 or 14 hours before the test; to eat nothing after this meal; to get a good night of rest; to eat no breakfast; and to dress and come to the laboratory in a leisurely manner, riding if possible. He must not exert himself. Upon arrival he is given absolute rest for 30 or 40 minutes before the test.

Wrap recording paper tightly around kymograph drum and paste in place either with a gummed strip or with paper stickers. Fill recording pen with ink.

By means of rubber tube, connect oxygen compression cylinder to the petcock on the apparatus for introducing oxygen gas. Admit oxygen into apparatus until pen is within a few centimeters of the edge of the paper drum. Shut valve on oxygen tank and petcock on apparatus. Connect patient with mouthpiece of apparatus and start motor. Adjust nose clip. Start kymograph revolving. Record temperature of gas in apparatus, and pulse of patient. At the end of 8 minutes remove nose clip and mouthpiece from patient. Stop motor and kymograph. Record temperature of gas in apparatus. Record barometric pressure. Determine patient's nude weight and his temperature. Record sex of patient. Record his age in years to nearest birthday.

**Calculation.**—Companies manufacturing apparatus supply direction booklets and tables for making calculations. In case a check on calculations is desired, the ap-

\* *Quantitative Clinical Chemistry.* Williams and Wilkins Co. Baltimore, Md.



parent volume of oxygen used by the patient can be converted to the true volume of dry gas at 0° C. and 760 mm. pressure by multiplying by the factor  $F$ , where  $F = 0.0012 \times B + 0.001 [4.6(30-t^\circ) - 52]$ .  $B$  is the observed barometric pressure in millimeters, and  $t^\circ$  is the observed temperature of the gas in degrees Centigrade.

Each liter of oxygen under standard conditions represents 4.825 calories of heat production (the respiratory quotient being assumed equal to 0.82). Therefore the heat production of the patient per hour is

$$C = \frac{F \times V \times 0.004825 \times 60}{M} \text{ Calories,}$$

where  $F$  has the value found above, and  $V$  is the apparent number of cc. of oxygen absorbed by the patient in  $M$  minutes.

The number of calories of heat which should be produced by an individual of the same biometric measurements can be found from the manufacturers' tables mentioned above, or in Peters and Van Slyke, Volume II, pp. 207-214. If the normal number of calories is  $N$ , the patient's basal metabolic rate is

$$\frac{C - N}{N} \times 100 \text{ per cent.}$$

*Notes.*—1. It is advisable in case the patient has never had a basal metabolic rate determination to make a practice run to accustom him to the procedure. This run may be made in the afternoon. Its results are of course discarded.

2. For accurate results the patient must be completely relaxed, and must make no movements during the determination.

3. The patient's temperature should be noted, as the metabolic rate rises about 6 per cent with each degree Fahrenheit of fever.

4. A basal metabolic rate between  $-10$  and  $+15$  cannot be considered definitely pathological.

5. The determination of basal metabolic rate is of clinical value principally in diagnosing and following the effect of treatment (surgical, radiological, or medical) of disorders of the thyroid gland. In hyperthyroidism the rate is above normal; in hypothyroidism the rate is below normal. Determinations may also be of value in disorders of some of the other glands of internal secretion.

# METHODS FOR THE CHEMICAL EXAMINATION OF MILK AND OTHER FOODS

Ordinarily milk examinations are made to determine the nutritive value and this information is obtained by estimating the specific gravity, total solids, ash, fat, protein, and lactose.

When foreign ingredients or adulterants are present in milk, special methods are employed to detect them.

## COLLECTION AND SAMPLING OF COW'S MILK

1. Great care is necessary to secure a homogeneous sample. In the case of bottled milk, collect one or more bottles as prepared for sale. In sampling bulk milk, thoroughly mix by pouring from one clean vessel into another 3 or 4 times. If this procedure is impracticable, thoroughly stir the milk for at least  $\frac{1}{2}$  minute with a suitable appliance long enough to reach to the bottom of the container. If cream has formed on the milk, continue the mixing until all cream is detached from the sides of the vessel and evenly emulsified throughout the liquid.

2. Place the samples in nonabsorbent, air-tight containers and keep them in the cold, but at a temperature above freezing, until ready for examination. When transported by mail, express, or otherwise, the containers should be completely filled, tightly stoppered, and marked for identification. A necessary quantity of preservative (corrosive sublimate, potassium dichromate, or formaldehyde) may be used, except where the presence of the preservative may be objectionable in connection with physical or chemical tests to be applied in addition to the determination of fat.

3. The quantity of sample required will depend upon the number of determinations to be made. For the usual analysis collect 250 to 500 cc. ( $\frac{1}{2}$  to 1 pint) of sample; for the fat determination only, 50 to 60 cc. (approximately 2 fluid ounces) will suffice.

4. Before withdrawing portions for analytical determinations, bring the sample to a temperature of 15° to 20° C. and mix thoroughly by pouring into a clean receptacle and back until a homogeneous mixture is assured. If lumps of cream do not completely disappear, warm the sample to about 38° C., mix thoroughly, then cool to 15° to 20° C. In case a measured volume is required in a determination, bring the temperature of the sample to 20° C. before pipeting.

## DETERMINATION OF SPECIFIC GRAVITY

This is most readily obtained with the aid of a hydrometer, accurately graduated within the limits of the widest possible variation in the specific gravity of milk. Hydrometers for special use with milk are known as lactometers. They usually have a thermometer combined with them and the readings should be made at the temperature specified on the lactometer (usually 60° F., 15.6° C.) by immersing the cylinder containing the milk in tap water.

## DETERMINATION OF TOTAL SOLIDS

A platinum dish is desirable, if not available porcelain may be used.

1. Weigh dish on the chemical balance.

2. Add about 5 cc. of the thoroughly mixed sample of milk and weigh again to

milligrams. This weighing should be done as rapidly as possible to avoid inaccuracies due to evaporation.

3. Place dish on a hot water bath until dry (about 2 hours) then for about 10 minutes in a hot air oven ( $100^{\circ}$  to  $105^{\circ}$  C.).

4. Cool to room temperature in a desiccator and weigh.

5. Multiply the weight of the residue by 100 and divide by the weight of the sample taken to obtain the per cent of total solids in the milk.

*Note.*—Prolonged heating in the drying oven should be avoided as this tends to decomposition. This is indicated by the formation of a brownish color. The residue should be nearly pure white.

### **DETERMINATION OF ASH**

1. The dish containing the milk residue (above) is placed on a triangle supported on a ring stand and ignited at a dull red heat by means of a Bunsen burner until a perfectly white ash is obtained.

2. Cool in a desiccator to room temperature and weigh.

3. Multiply the weight of the residue by 100 and divide by the weight of the original sample to obtain the per cent of ash in the milk.

### **BABCOCK METHOD FOR ESTIMATING THE FAT OF COW'S MILK**

**Principle.**—Strong sulphuric acid is added to the milk to dissolve the serum solids and set free the fat from its emulsion. The fat is then permitted to rise into the graduated neck of a Babcock bottle and the percentage read directly.

**Reagent and Apparatus.**—1. Commercial concentrated sulphuric acid with a specific gravity of 1.82 to 1.83 at  $20^{\circ}$  C.

2. Special Babcock milk pipet (Fig. 327) graduated to deliver 17.5 cc. but which holds 17.6 cc. to the graduation mark, the extra 0.1 cc. being allowed for the milk which clings to the walls.

3. Standard Babcock test bottle (Fig. 328).

4. Acid measure (Fig. 329) graduated to hold 17.5 cc.

5. Centrifuge for the Babcock bottles. Special trunnion cups may be purchased for use with the International Centrifuge.

**Procedure.**—1. Transfer 17.5 cc. (equivalent to 18 grams) of well-mixed milk to a milk test bottle by means of the special pipet. The milk remaining in the pipet tip is blown out.

2. Add 17.5 cc. of sulphuric acid, preferably not all at one time, pouring it down the side of the neck of the bottle in such a way as to wash any traces of the milk into the bulb. The temperature of the acid should be about  $15^{\circ}$  to  $20^{\circ}$  C.

3. Mix by rotation until all traces of curd have disappeared; then transfer the bottle to the centrifuge; counterbalance it; and, after the proper speed has been attained, whirl 5 minutes.

4. Add soft water at  $60^{\circ}$  C., or above, until the bulb of the bottle is filled.

5. Whirl 2 minutes.

6. Add hot water until the liquid column approaches the top graduation of the scale.

7. Whirl 1 minute longer at a temperature of  $55^{\circ}$  to  $60^{\circ}$  C. Transfer the bottle to the warm water bath maintained at a temperature of  $55^{\circ}$  to  $60^{\circ}$  C., immerse it to the level of the top of the fat column, and leave it there until the column is in equilibrium and the lower fat surface has assumed a final form.

8. Remove the bottle from the bath; wipe it; and measure the fat column, in terms of percentage by weight, from its lower surface to the highest point of the upper meniscus.



FIG. 327.—SPECIAL  
BABCOCK MILK  
PIPET



FIG. 328.—BABCOCK  
MILK TEST BOTTLE



FIG. 329.—A C I D  
MEASURE FOR  
THE BABCOCK  
METHOD



FIG. 330.—CREAM  
TEST BOTTLE

9. The fat column, at the time of measurement, should be translucent, of a golden yellow or amber color, and free from visible suspended particles. Reject all tests in which the fat column contains milk or shows the presence of curd or of charred matter, or in which the reading is indistinct or uncertain.

**Estimation of Fat in Cream.**—1. Because of variation in specific gravity of cream and its high viscosity, 18-gram samples can be accurately taken only by weighing, but with cream containing between 20 and 30 per cent fat the sample is sufficiently accurate for routine work by measuring 18 cc. of cream with a special pipet. Use the special cream test bottle shown in Figure 330.

2. Proceed as with milk except that readings are made at the bottom of the upper meniscus. Liquid petrolatum added will flatten out the meniscus and make the reading easier.

**Notes.**—1. In adding the acid, the test bottle is conveniently held at an angle so that the acid will run down the wall of the bottle and not in a small stream into the center of the milk, the bottle being slowly turned around and the neck thus cleared of adhering milk. The milk and the acid in the test bottle should be in two distinct layers without much of a black band of partially mixed liquids between them. Such a dark

layer is often followed by an indistinct separation of the fat in the final reading. The cause of this may be that a partial mixture of acid and milk before the acid is diluted with the water of the milk will bring about too strong an action of the acid in this small portion of the milk, and thus char the fat contained therein. The appearance of black flocculent matter in or below the column of fat renders a correct measurement difficult and at times even impossible; if the black specks occur in the fat column itself, the readings are apt to be too high; if below it, the difficulty comes in deciding where the column of fat begins.

2. The acid should be carefully mixed with the milk by giving the test bottle a rotary motion. In doing this care should be taken that the liquid is not shaken into the neck of the test bottle. When once begun the mixing should be continued until completed; a partial and interrupted mixing of the liquids will often cause more or less black material to separate with the fat when the test is finished. Clots of curd which separate at first by the action of the acid on the milk must be entirely dissolved by continued and careful shaking.

### ESTIMATION OF TOTAL NITROGEN OF COW'S MILK

**Principle.**—Organic compounds are oxidized and the nitrogen converted into ammonia which is distilled off into a standard acid solution and titrated with standard alkali solution.

**Procedure.**—1. Place 5 grams of the milk, weighed accurately, in a 500 cc. Kjeldahl digestion flask.

2. Add approximately 0.7 gram of mercuric oxide, or 0.1 gram of crystallized copper sulphate, 10 grams of potassium sulphate (or 10 grams of anhydrous sodium sulphate) and 25 to 30 cc. of sulphuric acid, specific gravity 1.84.

3. Place the flask in an inclined position and heat below the boiling point of the acid until frothing has ceased. (A small piece of paraffin may be added to prevent extreme frothing.) Then increase the heat until the acid boils briskly and digest for a time after the mixture is colorless or nearly so, or until oxidation is complete (Fig. 331).

4. Cool, dilute with about 200 cc. of water, cool again, add a few pieces of granulated zinc or pumice stone, if necessary to prevent bumping, and 25 cc. of a 4 per cent solution of potassium sulphide with shaking. (When no mercury or mercuric oxide is used the addition of the potassium sulphide solution is unnecessary.)

5. Next add sufficient saturated solution of sodium hydroxide free from nitrate, to make the reaction strongly alkaline (50 cc. are usually enough), pouring it down the side of the flask slowly and carefully so that it does not mix at once with the acid solution.

6. Connect the flask by means of a Kjeldahl connecting bulb with a condenser (Fig. 332), mix the contents of the flask by shaking, and distill until all ammonia has passed over into 50 cc. of standard N/10 hydrochloric or sulphuric acid. The first 150 cc. of the distillate will generally contain all the ammonia.

7. Titrate with N/10 standard alkali solution, using methyl red or cochineal solution as indicator.

8. Multiply the percentage of nitrogen by 6.38 to obtain the equivalent percentage to be reported as milk proteins.

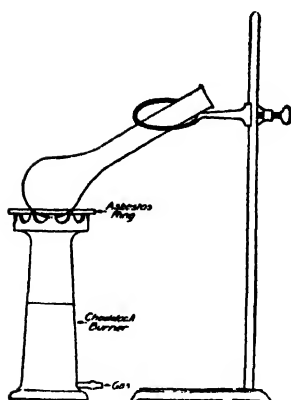


FIG. 331.—SUPPORT FOR  
KJELDAHL DIGESTION

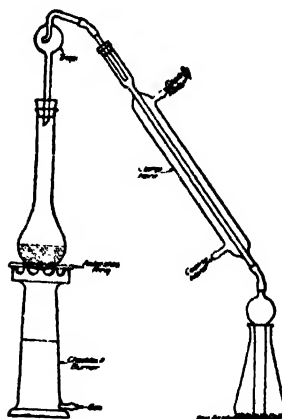


FIG. 332.—APPARATUS FOR THE  
DISTILLATION OF AMMONIA  
FOR NITROGEN DETERMINA-  
TION

#### Calculation:

$$\frac{50 - \text{cc. N/10 sodium hydroxide} - \text{cc. blank} \times 0.14}{\text{weight of sample}} = \text{per cent total nitrogen}$$

*Notes.*—1. Determinations are to be made in duplicate and blanks are to be run, using about 1 gram of cane sugar instead of the unknown. Sugar aids in the reduction of any nitrates that may be present in the reagents.

2. The flame of the burner should strike only the portion of the flask below the level of the acid. Sheet iron or asbestos board with a hole in it serves well as a support.

3. The Kjeldahl flask should be fitted with a rubber stopper through which passes the lower end of a Kjeldahl connecting trap bulb to prevent sodium hydroxide being carried over mechanically during the distillation. The bulb should be about 3 centimeters in diameter and the tubes should be of the same diameter as the condenser tube with which the upper end of the bulb is connected by means of rubber tubing. A piece of glass tubing about 12 centimeters long of the same diameter as the condenser tubing is attached to the lower end of the condenser by means of rubber tubing and should reach nearly to the bottom of the Erlenmeyer flask. This delivery tube is capable of being detached from the condenser for purposes of rinsing. It is preferable that the distilling system be made of Pyrex glass.

#### DETERMINATION OF CASEIN OF COW'S MILK

1. This determination should be made while the milk is fresh, or nearly so. When it is not practicable to make this determination within 24 hours, add 1 part of formaldehyde to 2500 parts of milk and keep in a cool place.

2. Place 10 grams of the sample in a beaker with 90 cc. of water at 40° to 42° C. and add at once 1.5 cc. of dilute acetic acid (1 + 9). Stir, and let stand 3 to 5 minutes.

3. Decant on a filter, wash by decantation two or three times with cold water, and transfer the precipitate to the filter. Wash once or twice on the filter.

4. The filtrate should be clear, or very nearly so. If the first portions of the filtrate are not clear, repeat the filtration, after which complete the washing of the precipitate.

5. Transfer without loss to a Kjeldahl flask and determine nitrogen in the washed precipitate and filter paper as described above for total nitrogen. Multiply by 6.38 to obtain the equivalent of casein.

6. To a sample of milk that has been preserved, the acetic acid should be added in small portions, a few drops at a time, with stirring, and the addition should be continued until the liquid above the precipitate becomes clear, or very nearly so.

### ESTIMATION OF MILK SUGAR

**Principle.**—The reducing action of the clarified milk is measured by a definite amount of Fehling's solution.

**Reagents.**—A. *Fehling's Copper Solution*—34.639 gms. C.P. copper sulfate are dissolved in water in a 500 cc. volumetric flask and the solution is then diluted to the mark.

B. *Fehling's Alkaline Tartrate Solution*—173 gms. Rochelle salts and 50 gms. sodium hydroxide are dissolved in water and diluted to 500 cc.

**Procedure.**—1. Place 25 gms. milk in a 250 cc. flask, add 0.5 cc. of 30 per cent acetic acid and shake well. After standing a few minutes about 100 cc. of boiling water are run in, the flask shaken and 25 cc. of alumina cream added. Let stand about 10 minutes. Pour through a ribbed filter paper, catching filtrate in a 250 cc. volumetric flask. Wash the precipitate thoroughly, allowing the washings to run into the volumetric flask which is then filled to the mark. The filtrate should be perfectly clear.

2. Fill a 50 cc. buret to the mark with this filtrate.

3. In a 250 cc. porcelain casserole place exactly 5 cc. each of solutions A and B. Add about 40 cc. of water and heat to boiling.

4. While still boiling add the milk filtrate from the buret, a small portion at a time until all the copper is reduced. This is indicated by a gradual color change from deep blue through green to dull red. The first appearance of this color is the end point.

5. Note from buret the volume of milk filtrate used.

**Calculation.**—Ten cc. of the above Fehling's mixture is equal to 0.067 gm. of milk sugar. The milk filtrate represents a tenfold dilution of the milk so that 0.067 multiplied by 10 and divided by the volume of filtrate used represents the per cent of lactose in the milk.

**Notes.**—1. Practice will soon enable the eye to judge the end point quite definitely.

2. The sugar-containing solution may be added quite rapidly until the solution becomes pale green, when the solution should be added cautiously, a few drops at a time.

### DETECTION OF PRESERVATIVES IN COW'S MILK

**Phenylhydrazin Test for Formaldehyde.**—1. To a portion of the sample add an equal volume of strong alcohol, shake and filter from any insoluble matter.

2. To 5 cc. of the filtrate add 0.03 gram of phenylhydrazin hydrochloride and 4 or 5 drops of a 1 per cent ferric chloride solution.

3. Mix, add slowly with agitation, in a bath of cold water to prevent heating the liquid, 1 to 2 cc. of concentrated sulphuric acid.

4. Dissolve the precipitate by the addition of either concentrated sulphuric acid (keeping the mixture cool) or alcohol. In presence of formaldehyde a red color develops.

**Ferric Chloride Test for Salicylic Acid.**—1. Acidify 100 cc. of the milk with 5 cc. of hydrochloric acid (1 + 3), shake until curdled, filter, and extract with 50 to 100 cc. of ether.

2. Wash the ether layer with two 5 cc. portions of water, evaporate the greater portion of the ether in a porcelain dish on a steam bath, allow the remainder to evaporate spontaneously, and add a drop of 0.5 per cent ferric chloride solution.

3. A violet color indicates salicylic acid.

**Test for Benzoic Acid.**—1. Acidify, filter, and extract a 100 cc. portion of the milk with ether as directed for salicylic acid. If benzoic acid is present in considerable quantity, it will crystallize from the ether in shining leaflets having a characteristic odor on heating.

2. Dissolve the residue in hot water, divide into 2 portions, and test as directed below. The residue may also be purified by sublimation and the melting point determined.

(a) Make the solution alkaline with ammonium hydroxide, expel the excess of ammonia by evaporation, dissolve the residue in water, and add a few drops of a neutral 0.5 per cent ferric chloride solution. A brownish precipitate of ferric benzoate indicates the presence of benzoic acid.

(b) Add to the water portion 1 or 2 drops of a 10 per cent solution of sodium hydroxide and evaporate to dryness. To the residue add 5 to 10 drops of concentrated sulphuric acid and a small crystal of potassium nitrate. Heat for 10 minutes in a glycerol bath at 120° to 130° C., or for 20 minutes in a boiling water bath. The temperature must not exceed 130° C. After cooling add 1 cc. of water and make distinctly ammoniacal; boil the solution to decompose any ammonium nitrate that may have been formed. Cool and add a drop of fresh, colorless ammonium sulphide, without allowing the layers to mix. A red-brown ring indicates benzoic acid. On thorough mixing, the color readily diffuses through the whole liquid and, on heating, gradually and finally changes to greenish-yellow. This differentiates benzoic acid from salicylic acid or cinnamic acid. The last two form colored compounds, which are not destroyed by heating.

**Test for Borax and Boric Acid.**—1. Immerse a strip of turmeric paper in the sample acidified with hydrochloric acid in the proportion of 7 cc. of strong acid to each 100 cc. of sample and allow the paper to dry spontaneously.

2. If borax or boric acid is present, the paper will acquire a characteristic red color, changed by ammonium hydroxide to a dark blue-green, but restored by acid.

## COLLECTION AND CHEMICAL ANALYSIS OF HUMAN MILK

**Collection.**—There are 2 methods of obtaining samples of breast milk for analysis:

1. Express all the milk from 1 breast and mix thoroughly.

2. Draw 1 ounce of milk before nursing and 1 ounce after nursing. Mix the 2 samples thoroughly. The best time for obtaining samples is 9 to 10 A.M.

**Determination of Specific Gravity.**—Determined most conveniently by means of a Soxhlet, Veith or Quevenne lactometer at 60° F. The lactometer reading is cor-



rected by adding 0.0001 for every degree F. above 60° and subtracting 0.0001 for every degree F. below this temperature.

**Determination of Percentage of Fat.**—This is essentially a modification of the Babcock test previously described except that a smaller bottle is employed (Fig. 333); the technic is practically the same. Otherwise the regular Babcock bottle and method are to be preferred if sufficient sample is available.

1. By means of a special narrow pipet, introduce milk up to the 5 cc. mark.

2. Add sufficient commercial sulphuric acid (specific gravity 1.83) to fill the body of the tube and rotate to secure a homogeneous mixture.

3. Fill the neck of the tube with a mixture consisting of equal volumes of amyl alcohol and concentrated hydrochloric acid and centrifuge for 2 minutes, etc., as described above.

4. The fat collects in a column in the upper part of the acid alcohol mixture and the percentage is read off directly on the graduated stem.



FIG. 333.—BABCOCK  
FAT BOTTLE FOR  
HUMAN MILK

**Determination of Total Solids.**—1. Introduce 2 to 5 grams of milk into a weighed flat-bottomed platinum dish and quickly ascertain the weight to milligrams.

2. Carefully expel most of the water by heating in a water bath and heat for about 5 minutes in an oven regulated to a temperature between 100° and 105° C. Cool and weigh.

3. Divide the weight of the residue in grams by the weight of the milk used. Multiply the result by 100 to give the per cent of total solids in the milk.

**Determination of Ash.**—1. Heat the dry solids obtained above over a low flame (care should be taken that the dish is not heated above dull redness) until a white or light gray ash is obtained.

2. Cool the dish in a desiccator and weigh.

3. Divide the weight of the ash by the weight of the original milk taken for total solids and multiply by 100 to obtain the per cent of ash in the milk.

**Determination of Total Nitrogen (Proteins).**—1. Introduce 5 grams of milk into a 500 cc. Kjeldahl flask and carry out the nitrogen determination as described for cow's milk.

2. Multiply the per cent of nitrogen by the factor 6.37 to obtain the protein content of the milk.

**Determination of Lactose.**—1. Introduce 1 cc. of milk into a 100 cc. volumetric flask.

2. Add 2 cc. of 10 per cent sodium tungstate.

3. Add gradually 2 cc. of two-thirds normal sulphuric acid or 16 cc. of N/12 acid, mix well and let stand 5 minutes.

4. Dilute to the mark, mix and filter.

5. Into a Folin-Wu sugar tube introduce 1 cc. of the filtrate and add 1 cc. of water. Into another tube place 2 cc. of standard lactose solution. Add 2 cc. of the Folin-Wu alkaline copper reagent (see page 810) to each tube and heat in boiling

water for 8 minutes. Cool and add 2 cc. of phosphomolybdic reagent to each tube. Dilute to 25 cc. mark and compare in colorimeter.

6. Calculate as follows:

$$\frac{20}{R} \times 0.6 \times \frac{100}{0.01} \times \frac{1}{1000} = \text{per cent lactose} = \frac{120}{R} = \text{per cent lactose}$$

7. For the standard lactose solution, prepare a stock lactose solution by dissolving 1 gram of lactose (weighed on an analytical balance) in 0.2 per cent benzoic acid and making up to 100 cc. in a volumetric flask. The working standard is prepared by diluting 3 cc. of this stock solution in a 100 cc. volumetric flask to the mark with 0.02 per cent benzoic acid. Two cc. of this solution equal 0.6 milligram lactose.

## CHEMICAL ANALYSES OF DIABETIC FOODS

**Preparation of Sample.**—Grind sufficiently fine in a mortar or mill to pass through a 1 millimeter sieve. Preserve in a sealed container to prevent moisture changes.

**Detection of Starch.**—Starch is best detected, when present to any appreciable extent in any mixture, by boiling a portion of the sample in water, cooling and applying a solution of iodine. A characteristic blue color appears if starch is present. Very small amounts of starch are best identified by adding iodine solution to powder on a microscopic slide, or better, to the powder previously rubbed with water on a slide under a coverglass; the starch granules, if present, will be colored intensely blue by the iodine, and are at once rendered apparent when viewed through the microscope.

**Estimation of Moisture.**—Weigh accurately 2 to 3 grams of foodstuffs on a tared watch glass and dry to constant weight in an oven at 105° to 106° C. This requires about 4 hours. The loss in weight represents moisture.

**Estimation of Ash.**—Follow the procedure for determining the ash of human milk.

**Estimation of Protein.**—Determine the total nitrogen of a 1 gram sample according to the procedure given for the total nitrogen of milk. Calculate the protein by multiplying the total nitrogen by the appropriate factor which varies with different cereals as follows: wheat, 5.70; rye, 5.62; oats, 6.31; corn, 6.39; and barley, 5.82. If the sample represents a mixture of various grains the conventional factor 6.25 is employed.

**Estimation of Fat.**—Heat on a boiling water bath for 90 minutes 5 grams of the sample in a 200 cc. flask with 50 cc. of water and 2 cc. of 25 per cent hydrochloric acid (specific gravity 1.125). Cool, nearly neutralize with 40 per cent sodium hydroxide (using 0.04 per cent methyl orange as indicator) and filter. Transfer contents to a filter paper and wash with hot water. Dry filter and contents in oven at 105° C. and transfer to extraction thimble of Soxhlet extractor. Extract with ether for 16 hours, transfer the extract to a weighed beaker, evaporate the ether on the water bath and dry to constant weight in the oven. This increase in weight represents the fat.

**Estimation of Starch, Sugar and Dextrin.**—1. Extract 4 to 5 grams of the fine powder (accurately weighed) on a hardened filter paper with five successive 10 cc. portions of ether.

2. Wash the powder into a beaker with 50 cc. of water.

3. Immerse the beaker in a boiling water bath for 15 minutes or until all the starch is gelatinized.

4. Cool to 55° C.

5. Add 20 cc. of a fresh 0.5 per cent aqueous solution of U.S.P. pancreatin.

6. Digest at 55° C. for 1 hour.

7. Heat again to boiling for a few minutes to gelatinize the remaining starch granules.

8. Cool to 55° C. and redigest at this temperature with another 20 cc. portion of pancreatin solution for 1 hour or until the residue treated with iodine gives no test for starch.

9. Cool, make up to 250° C. and filter.

10. Place 200 cc. of the filtrate into a flask, add 20 cc. of dilute hydrochloric acid (specific gravity 1.125; made by adding 2 volumes of water to 5 volumes of concentrated acid) connect with a reflux condenser and heat in a boiling water bath for 2½ hours.

11. Cool, nearly neutralize with 10 per cent sodium hydroxide, finish the neutralization with sodium carbonate and dilute to 500 cc.

12. Mix the solution, pour through a dry filter and determine the dextrose in an aliquot part according to Benedict's method for sugar in urine.

*Calculation:* The amount of dextrose found multiplied by 0.9 gives the amount of starch. This amount multiplied by the aliquot part taken and the 2 dilutions gives the starch in the original sample from which the percentage may then be calculated.

# METHODS OF EXAMINATION FOR VITAMINS IN BLOOD AND URINE

## METHOD FOR DETERMINATION OF BETA-CAROTENE AND VITAMIN A IN THE BLOOD

**Principle.**—In this modified colorimetric method of Pett and LePage (*Jour. Biol. Chem.*, 132: 585, 1940) the serum is treated with alcohol, extracted with petroleum ether and the amount of yellow color, measured in the photoelectric colorimeter, is calculated to beta-carotene. The petroleum ether extracted material is then treated with antimony trichloride solution, the blue color measured in the photoelectric colorimeter and equivalent vitamin A calculated after correcting for the color production due to beta-carotene.

**Reagents.**—*Alcohol, 95 per cent.*

*Petroleum ether, B.P., 35° to 60° C.*

**Chloroform.**—Reagent chloroform is dried over anhydrous potassium carbonate and distilled in an all glass still under reduced pressure at 30° to 35° C., the first 25 cc. of the distillate being discarded.

**Antimony trichloride solution.**—Place 22 gm. of pure, fresh reagent antimony trichloride in a 100 cc. glass stoppered cylinder. Add the dry, redistilled chloroform to the 100 cc. mark and shake at intervals. Complete solution takes place in about 24 hours. Protect from light.

**Artificial Beta-Carotene Standard.**—Dissolve 200 mg. of potassium dichromate in water, transfer to a 1-liter volumetric flask and dilute to the mark with water. The color is equivalent to 5.6 microgm. of beta-carotene per 5 cc. of petroleum ether extract.

**Artificial Vitamin A Standard.**—Prepare an accurate 5 per cent solution of copper sulfate. The color is equivalent to 9.2 international units of vitamin A per 5 cc. of solution.

**Procedure for Carotinoids.**—1. Place 5 cc. of serum in a test tube of about 30 cc. capacity, add 5 cc. of alcohol and 10 cc. of petroleum ether, stopper the tube and shake thoroughly for at least 5 minutes. Remove the stopper, cap the tube and centrifuge.

2. Pipet the clear ether layer into a Klett-Summerson colorimeter tube graduated at 5 cc. and reduce the volume to the mark by means of gentle warming and a current of air.

3. Stopper the tube and read in the colorimeter using light filter No. 44 and a petroleum ether zero setting.

4. Determine the colorimeter reading of the standard dichromate solution using the same color filter but with a distilled water zero setting.

**Calculation.**—
$$\frac{112}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{beta-}$$

carotene in microgm. per 100 cc. of serum.

**Procedure for Vitamin A.**—1. Replace color filter No. 44 with filter No. 62 and adjust the zero setting with a tube of antimony trichloride solution.

2. Place the colorimeter tube containing the petroleum ether extract in a water bath at 50° C. and evaporate the solvent completely with the aid of a stream of air.

Raise the temperature to about 70° C. for a moment or two at the end of the evaporation.

3. Dissolve the residue in 0.5 cc. of chloroform and insert the tube in the colorimeter.

4. Using a 5 cc. pipet, with a tip sufficiently large to deliver completely in about 2 seconds, add 4.5 cc. of the antimony trichloride solution as rapidly as possible.

5. Read the color value at once as the color produced is evanescent.

6. Determine the colorimeter reading of the standard copper sulfate solution using the same color filter but a distilled water zero setting.

*Calculation.*—  $\frac{184}{\text{reading of standard}}$  (= factor)  $\times$  reading of unknown = vitamin

A plus beta-carotene in I.U. per 100 cc. of serum.

Pett and LePage state that 88 micrograms of beta-carotene produce a blue color equivalent to 84.1 international units of vitamin A. Therefore, multiply the micrograms of beta-carotene previously found for the serum by 0.96 and subtract this result from the above to give the vitamin A content in international units per 100 cc.

*Notes.*—1. A more accurate color reading for vitamin A is obtained by using duplicate samples; the initial reading being made from one sample after which the colorimeter tube containing the duplicate sample is inserted without changing the colorimeter setting. The addition of the antimony trichloride reagent to this duplicate sample will produce a galvanometer swing that will be nearly balanced by the first reading; any loss of color that had been missed in the first reading due to fading is noted in the duplicate, the reading of this second sample being taken as the true reading.

2. Saponification of the serum prior to petroleum ether extraction at times gives an increased yield of chromogenic substances. Whether this is actually vitamin A is in some doubt and this step has been omitted in the above procedure.

3. Normal vitamin A values range from 40 to 60 international units per 100 cc. of blood. Levels below 30 units are indicative of an unsatisfactory nutritional state.

## METHOD FOR DETERMINATION OF THIAMIN IN URINE

**Principle.**—In this method adapted after that of Hennessy and Cercedo (*Jour. Am. Chem. Soc.*, 61: 179, 1939) the thiamin ( $B_1$ ), adsorbed on activated permutit and eluted with potassium chloride, is oxidized by ferricyanide in alkaline medium. The resulting thiochrome is extracted with isobutanol and the intensity of the violet-blue fluorescence estimated in a fluorocomparator.

**Adsorption Tube.\***—A glass tube of 7 mm. inside diameter is used. A constriction at the lower end holds a plug of glass wool. An enlarged portion is sealed on at the upper end for introduction of liquids.

**Reagents.**—Sodium hydroxide solution, 15 per cent.

Glacial acetic acid.

Potassium chloride solution, 25 per cent.

Potassium ferricyanide solution, 10 per cent.

Isobutyl alcohol, C.P.

\* May be obtained from E. Machlett and Son, New York City.

*Permutit, activated.*—Place 50 gm. of Decalso,\* 60 to 80 mesh, in a 500 cc. beaker. Add 100 cc. of the 25 per cent solution of potassium chloride and 5 cc. of glacial acetic acid. Heat to boiling and boil gently for 30 minutes with constant stirring. Allow the powder to settle and decant the supernatant. Repeat this treatment, then wash the permutit on a filter with 2 liters of boiling distilled water. Transfer the moist powder to an evaporating dish and dry at 100° C. Keep in a tightly stoppered bottle for future use.

**Procedure.**—1. Urine for thiamin determinations should be kept in a dark bottle preserved with 3 per cent of its volume of glacial acetic acid.

2. Five gm. of “activated” permutit is suspended in a little water and poured into the adsorption tube. Allow the excess water to drain off.

3. Heat 10 cc. of the urine to boiling and pour while hot into the adsorption column.

4. When the urine has filtered through, wash the column with 100 cc. of boiling water and dry by gentle suction.

5. Pour 15 cc. of boiling 25 per cent potassium chloride solution into the adsorption column at such a rate that the elution process takes a total of at least 10 minutes, carefully collecting all of the eluate that appears at the constricted portion of the tube.

6. Introduce 10 cc. of this eluate into a separatory funnel; add 6 cc. of 15 per cent sodium hydroxide, 0.2 cc. of 1 per cent potassium ferricyanide and 5 cc. of isobutyl alcohol.

7. Shake vigorously for 2 minutes and allow the 2 layers to separate.

8. Drain off and discard the lower watery layer.

9. Transfer the upper turbid isobutyl alcohol layer to a test tube, cap the tube and centrifuge for 5 minutes.

10. Pipet off 3 or 4 cc. into a standard test tube 1 cm. in diameter and examine for fluorescence in a fluorocomparator,\*\* the fluorescence being matched against a series of stable standards.

**Notes.**—1. Patients to be tested should not be receiving drugs, since a number of drugs cause the appearance of fluorescent substances in urine which may interfere with the determination.

2. The presence of thiochrome fluorescence in a urine specimen obtained after the morning voiding, but before breakfast, indicates that the individual is adequately supplied with thiamin.

3. While the permutit in the adsorption column can be reactivated following use, it is advisable to use a fresh portion for each determination.

## METHOD FOR DETERMINATION OF PYRUVIC ACID IN BLOOD AND URINE

**Principle.**—In this method by Friedemann and Hangen (*Jour. Chem.*, 147: 415, 1943) the pyruvic acid is extracted with ethyl acetate and the red color produced by the action of 2, 4-dinitrophenylhydrazine on this extract in alkaline solution is measured in the photoelectric colorimeter.

\* May be obtained from The Permutit Company, New York City.

\*\* May be obtained from W. A. Taylor and Company, Baltimore, Md.

**Reagents.**—*Trichloroacetic acid solution 10 per cent*: Prepare at frequent intervals and keep in refrigerator when not in use. Old solutions yield increased blanks.

*Ethyl acetate, C.P., anhydrous.*

*Sodium carbonate solution, 10 per cent.*

*Sodium hydroxide solution, approximately 1.5 N.*

**Hydrazine reagent.**—Grind 100 mg. of 2, 4-dinitrophenylhydrazine (Eastman) in a mortar with increasing small quantities of approximately 2 N hydrochloric acid until 100 cc. have been added. Filter. Keep in refrigerator.

**Pyruvic Acid Standard.**—Pyruvic acid is redistilled in vacuo, the fraction boiling at 55° to 60° C. (10 mm. Hg.) is used. Add sufficient sulfuric acid to bring the final acidity to approximately 0.1N. Stored in the refrigerator, it keeps at least 6 months.

**Preparation of Standard Curve.**—A series of dilutions of pyruvic acid are freshly prepared containing from 0.25 to 6 mg. per 100 cc. Five cc. amounts of these dilutions are added to 25 cc. portions of the 10 per cent trichloroacetic acid, mixed, and treated as follows:

1. Place 3 cc. portions in 18 x 150 mm. test tubes.
2. Warm 10 minutes at 25° C., add 1 cc. of the hydrazine reagent, mix, and allow to stand at this temperature 5 minutes longer.
3. Add exactly 8 cc. of ethyl acetate and mix by means of a current of nitrogen or air blown in through a capillary pipet for 2 minutes.
4. Allow the layers to separate, tapping the tube to dislodge any globules of solution, and remove and discard the lower aqueous layer by means of the same pipet.
5. Add exactly 6 cc. of the sodium carbonate solution to the ethyl acetate portion and mix by again passing a current of gas through the mixture for 2 minutes using a capillary pipet.
6. Allow the layers to separate and remove and discard most of the ethyl acetate layer.
7. Withdraw 5 cc. of the aqueous layer, wipe the sides and tip of the pipet free of adherent ethyl acetate and transfer to a colorimeter tube.
8. Add exactly 5 cc. of the 1.5N sodium hydroxide solution and mix.
9. Using color filter No. 52, read in the colorimeter after 10 minutes against a zero prepared from 3 cc. of trichloroacetic acid solution which has been treated exactly as the standards above.
10. Plot the colorimeter readings against the corresponding pyruvic acid values on cross-section paper.

**Collection of Blood Sample.**—Use a cold 2 cc. tuberculin syringe. Withdraw from the vein 2 cc. of blood and transfer directly into 10 cc. of cold trichloroacetic acid solution contained in a 15 cc. centrifuge tube. Stopper the tube, shake, and after 10 minutes separate the protein by centrifuging.

**Collection of Urine Sample.**—Collect a 24-hour specimen of urine in a cold bottle containing 5 cc. of concentrated sulfuric acid. Collect single specimens in cold containers to which are added 0.5 of concentrated sulfuric acid for each 100 cc. of urine. Add 2 cc. of urine to 10 cc. of the trichloroacetic acid solution and centrifuge if necessary.

**Procedure.**—Treat 3 cc. of the blood supernatant or 3 cc. of the diluted urine exactly as described for the standards, carrying through also a water blank. Calculate the pyruvic acid content of the unknown by interpolation from the standard curve.

**Notes.**—1. If it is inconvenient to take the blood specimen as outlined, it is possible to prevent loss of pyruvic acid by preserving with iodoacetic acid as follows: A solution of 50 per cent iodoacetic acid in water is adjusted to pH 7.8 with sodium hydroxide and diluted with sufficient water to make a 25 per cent solution. Transfer 0.1 cc. of this solution to a bottle containing 20 mg. of oxalate. This is sufficient to preserve about 5 cc. of blood. In either case blood should be collected with a minimum of stasis and the patient should not be directed to open and close the hand in order to distend the vein. A warm syringe should not be used.

2. Normal blood contains about 1 mg. per cent of pyruvic acid. Normal urine may contain up to about 2 mg. per cent.

3. Blood pyruvic acid is increased (to about 3 mg. per cent) in thiamin deficiency.

### METHOD FOR DETERMINATION OF RIBOFLAVIN IN URINE

**Principle.**—In this method of Najjar (*Jour. Biol. Chem.*, 141: 355, 1941) the fluorescent compound formed from riboflavin by the action of pyridine in acid solution is extracted with normal butanol and estimated in a fluorocomparator.\*

**Reagents.**—*Acetic acid, glacial.*

*Pyridine, C.P.*

*Potassium permanganate solution, 4 per cent.*

*Hydrogen peroxide solution, 3 per cent.*

*Sodium sulfate, anhydrous.*

*Normal butyl alcohol, C.P.*

**Procedure.**—1. Urine for riboflavin determinations must be kept in dark bottles, preserved with about 3 per cent of its volume of glacial acetic acid; throughout the manipulations exposure to light should be avoided as far as possible.

2. The quantity of urine taken for analysis should be 5 cc. if a deficiency is suspected; normally 5 cc. of a 1:5 dilution is used.

3. In a 25 cc. glass stoppered cylinder place 5 cc. of diluted or undiluted urine; add 1 cc. of glacial acetic acid and 1 cc. of pyridine. Stopper the cylinder and shake vigorously.

4. Add 2 drops of 4 per cent permanganate for each cc. of undiluted urine used. Let stand 2 minutes.

5. Add sufficient 3 per cent peroxide to decolorize the solution.

6. Add 1 gm. of anhydrous sodium sulfate for each cc. of undiluted urine used and then 5 cc. of the n-butanol. Stopper the cylinder and shake vigorously for 2 minutes.

7. Transfer to a centrifuge tube, cap the tube, and centrifuge at low speed for 10 minutes to separate the layers.

8. Pipet about 4 cc. of the clear pyridine butanol layer into a standard comparator tube.

9. Match the fluorescence against the riboflavin standards in the fluorocomparator.

**Note.**—The presence of fluorescence in a urine specimen obtained after the morning voiding, but before breakfast indicates that the individual is adequately supplied with riboflavin.

\* May be obtained from W. A. Taylor & Co., Baltimore, Md.



**METHOD FOR DETERMINATION OF PANTOTHENIC ACID IN BLOOD**

**Principle.**—In this method described by Pennington, Snell and Williams (*Jour. Biol. Chem.*, 135: 213, 1940) and by Stanberg, Snell and Spies (*Jour Biol. Chem.*, 135: 353, 1940) the growth response to the blood pantothenic acid of a lactic acid producing organism in a synthetic pantothenic acid-free medium, is measured by the change in pH after 24-hour incubation.

**Organism.**—The organism is *Lactobacillus casei* E.\* Stock cultures are carried as stabs in yeast extract-glucose-agar (1 per cent glucose, 1 per cent yeast extract, 1.5 per cent agar). These stabs are prepared from previous stock cultures at monthly intervals. After transfer, cultures are incubated at 37° C. for 24 to 48 hours, then held in the refrigerator. Inoculum for assay tubes is prepared by transfer from the stock culture to a sterile tube of the basal medium (described below) to which an excess of pantothenic acid (or pantothenic acid-containing extract, e.g., yeast extract) has been added. The inoculum is incubated at 37° C. for 24 hours before use. For tests on successive days, the inoculum for the following day may be prepared from that of the preceding day.

**Basal Medium.**—This consists of alkali-treated peptone 0.5 per cent, glucose 1 per cent, sodium acetate 0.6 per cent, alkali-treated yeast extract equivalent to 0.1 per cent yeast extract, acid hydrolyzed casein equivalent to 0.2 per cent, cystine 0.01 per cent, riboflavin 0.01 mg. per cent, and inorganic salts. The constituents of the medium are prepared as follows:

**Alkali-treated Peptone.**—Forty gm. of Bacto-Peptone (Difco) in 250 cc. of water are treated with 20 gm. of sodium hydroxide dissolved in 250 cc. of water. The mixture is allowed to stand at room temperature for 24 hours. Neutralize with glacial acetic acid (27.9 cc.), add 7 gm. of anhydrous sodium acetate and dilute to 800 cc. Preserve under toluene.

**Cystine.**—A solution of cystine hydrochloride to contain 1 mg. of cystine per cc. is preserved under toluene.

**Alkali-treated Yeast Extract.**—Dissolve 20 gm. of Difco yeast extract in 200 cc. of 0.5N sodium hydroxide. Autoclave at 15 lbs. pressure for 30 minutes. Neutralize the cooled solution with glacial acetic acid, autoclave 10 minutes and filter. (This treatment destroys the pantothenic acid which is normally present in yeast extract so that it is necessary, as previously indicated, to add untreated yeast extract to the basal medium when making subcultures of the organism for use in the assay.) Adjust the volume to 200 cc. and preserve under toluene.

**Acid-Hydrolyzed Casein.**—Add 250 cc. of 25 per cent sulfuric acid to 50 gm. of technical casein. Mix and autoclave 10 hours at 15 pounds. Remove the sulfuric acid by adding barium hydroxide (341 gm. of Ba (OH)<sub>2</sub> 8H<sub>2</sub>O). Filter. Remove the slight excess of barium with the cautious addition of sulfuric acid. Filter. Adjust the pH to 7.0 with sodium hydroxide solution. Make up to 450 cc., and autoclave for 10 minutes. Preserve under toluene.

**Inorganic Salts.**—Solution A: 25 gm. of potassium monohydrogen phosphate and 25 gm. of potassium dihydrogen phosphate dissolved in 250 cc. of water.

Solution B: 10 gm. of magnesium sulfate heptahydrate, 0.5 gm. of sodium chloride,

\*Cultures of this organism may be obtained from the American Type Culture Collection, Georgetown University Medical School, Washington, where it is listed as No. 7469.

0.5 gm. of ferrous sulfate heptahydrate, and 0.5 gm. of manganese sulfate tetrahydrate dissolved in 250 cc. of water. Salts precipitate from this solution on standing in air; it need be renewed only when a uniform suspension can no longer be secured by shaking.

5 cc. of each solution are used for 1 liter of basal medium.

**Riboflavin.**—A solution of riboflavin in 0.02N acetic acid to contain 10 microgm. per cc. Keep under toluene in the refrigerator. Avoid unnecessary exposure to light and prepare fresh at frequent intervals.

**Pantothenic Acid Standard.**—A solution of 0.03 microgm. per cc. made from pure calcium pantothenate (Merck) or an assayed yeast extract suitably diluted.

**Procedure.**—1. Mix 10 cc. of the alkali-treated peptone, 2 cc. of the casein hydrolysate, 1 cc. of the alkali-treated yeast extract, 10 cc. of the cystine hydrochloride solution, 0.5 cc. of solutions A and B, 1 cc. of the riboflavin solution and 1 gm. of glucose. Adjust the pH to 6.8-7.0 and dilute to 50 cc. This is twice the concentration needed for the final medium, since in the assays it is mixed with an equal volume of unknown.

2. Take 1 cc. of citrated blood with 9 cc. of water and place a 2 cc. and a 3 cc. portion into 19 x 150 mm. test tubes.

3. In 6 other similar test tubes place 0.5, 1.0, 2.0, 3, 4 and 5 cc. of the standard pantothenic acid solution.

4. Dilute all 8 tubes to 5 cc. with water if necessary.

5. Add to each tube 5 cc. of the pantothenic acid-free basal medium, plug the tubes with cotton and autoclave 15 minutes at 15 pounds pressure.

6. Shake the 2 tubes containing the blood sample to break up the precipitated proteins and inoculate all tubes as follows: The cells from a 24-hour culture of inoculum, grown as previously described on the basal medium with added pantothenic acid, are aseptically thrown down by centrifuging and resuspended in approximately twice the original volume of 0.9 per cent sterile saline solution. One drop of the resulting suspension of organisms is added to each tube. Variations in the size of the drop do not cause appreciable errors in the final results.

7. Place all tubes in the incubator (37° to 38° C.) for 24 hours.

8. Inhibit the growth in all of the tubes simultaneously by chilling (in a refrigerator or preferably in a beaker of ice water) until ready for the determination of pH, at which time they are brought to 25° C. by immersing in warm water.

9. Determine the pH of the tubes containing the standards (a Beckman pH meter is convenient) and plot the results against the corresponding pantothenic acid concentrations on cross-section paper, drawing a smooth curve through the points. Such a chart, once completed, may be used for future assays so that standards need not be run with all unknowns.

10. Determine the pH of the 2 tubes containing the blood sample and pick off from the chart the corresponding amounts of pantothenic acid.

11. Divide each figure by the amount of blood used in that tube (*i.e.*, 0.2 or 0.3 cc.) and average the result. Multiply the average by 100 for the pantothenic acid content in microgm. per 100 cc.

**Notes.**—1. Pantothenic acid in blood of normal individuals varies between 18 and 35 micrograms per 100 cc.; average about 23.

2. The blood of patients with pellagra, beriberi and riboflavin deficiency show a decreased pantothenic acid content averaging 23 to 50 per cent below normal average.

### METHOD FOR DETERMINATION OF BIOTIN IN BLOOD AND URINE

**Principle.**—In this method by Shull, Hutchings and Peterson (*Jour. Biol. Chem.*, 142: 913, 1942) the growth response to the biotin in blood or urine of a lactic acid producing organism, in a synthetic biotin-free medium, is measured after 24-hour incubation by the change in pH.

**Organism.**—The organism is the same as that used in the assay for pantothenic acid described above.

The inoculum for the assay is started 2 days before the assay is set up. A transfer is made from a stab culture to 10 cc. of a tube of liquid medium containing 0.5 per cent of yeast extract (Difco), 0.6 per cent sodium acetate, 0.5 per cent glucose and 0.05 cc. each of the mineral salt solutions A and B (described below). The culture is incubated for 24 hours at 37° C. and then transferred to the sterile biotin-free basal medium described below. A 2 per cent inoculum is used for the transfer, *e.g.*, 0.2 cc. per 10 cc. of basal medium. This inoculum supplies sufficient biotin for a good growth of the organism after about 20 hours at 37° C. One drop of the suspension of this organism is used to inoculate each tube in the assay.

**Basal Medium.**—It is prepared from the constituents treated as follows:

**Peroxide-Treated Hydrolyzed Casein.**—To 100 gm. of Labco vitamin-free casein add 375 cc. of water and 125 cc. of concentrated sulfuric acid. Hydrolyze for 15 hours at 120° C. in an autoclave. Suspend 640 gm. of barium hydroxide in 400 cc. of boiling water and add to the hydrolysate. Filter off the barium sulfate with the aid of Filter Cel. Break up the filter cake in 500 cc. of hot water, filter and add the washings to the main filtrate. Adjust to pH 3.0 with sodium hydroxide solution, dilute to 1500 cc. and add 15 cc. of 30 per cent hydrogen peroxide. Stir and let stand 24 hours at room temperature. Adjust the pH to 7.0 with sodium hydroxide solution and add manganese dioxide powder in an amount equivalent to 1 per cent. Stir the mixture mechanically until no more oxygen is evolved (about 15 minutes) and filter with suction. Dilute the filtrate to 2 liters with water.

**Peroxide-Treated Norit Yeast Filtrate.**—Dissolve 20 gm. of Difco Bacto-yeast extract in 1 liter of water and adjust the pH to 2.0 with concentrated sulfuric acid. Add 4 gm. of norit A and stir mechanically for 30 minutes at 55° to 60° C. Remove the norit by filtration with the aid of Filter Cel and adjust the filtrate to pH 3.0 with 40 per cent sodium hydroxide. Make up the volume to 1300 cc., add 13 cc. of 30 per cent hydrogen peroxide and allow to stand at room temperature for 24 hours. Adjust the pH to 7.0 with 40 per cent sodium hydroxide and add 13 gm. of manganese dioxide. Stir mechanically until the evolution of oxygen ceases. Remove the manganese dioxide by filtration. The final volume should be about 1325 cc.

**Vitamin Solution.**—A solution containing 100 microgm. of pyridoxine, 100 microgm. of calcium pantothenate, 50 microgm. of riboflavin, 500 microgm. of nicotinic acid, and 50 microgm. of para-aminobenzoic acid per cc. is stored in the refrigerator under toluene.

**Adenine-Guanine-Cystine Solution.**—A solution containing 500 microgm. each of

adenine and guanine, and 1.0 mg. of cystine per cc. is prepared by dissolving these in water with the addition of the least amount of sulfuric acid needed to effect solution.

**Mineral Salt Solutions.**—Both solution A and B have the same composition as used in the medium for the determination of pantothenic acid, page 872.

It is convenient to prepare a quantity of stock basal medium complete, except for glucose, sodium acetate and mineral salts, in advance. Mix 200 cc. each of the peroxide-treated casein solution, the peroxide-treated yeast filtrate, and the adenine-guanine-cystine solution. Add 4 cc. of the vitamin solution and 150 mg. of tryptophane and 400 mg. of asparagine. Dilute to 1 liter and preserve under toluene in the refrigerator.

**Procedure.**—1. In a 50 cc. mixing cylinder, place 1 gm. each of C.P. anhydrous glucose and anhydrous sodium acetate, add 0.5 cc. each of mineral salt solutions A and B and dilute to 50 cc. with the stock basal medium. Adjust the pH of the complete biotin-free basal medium to 5.6. This mixture is sufficient for 10 assays.

2. Place 5 cc. portions of this medium in 15 x 150 mm. test tubes, add the solutions to be assayed and make up the final volume to 10 cc. with distilled water. Plug the tubes with cotton and autoclave for 15 minutes at 120° C. Cool and inoculate the tubes with 1 drop of the inoculum as described above. Incubate at 37 to 38° C. for 24 hours and chill the tubes by placing them in the refrigerator or in ice water until ready to determine the pH. This is most conveniently done by means of the glass electrode.

3. **Preparation of Standard Curve.**—A solution of biotin in the form of the free acid or the hydrolyzed methyl ester containing 200 micromicrograms per cc. is used. To 6 tubes containing 5 cc. of the basal culture medium add amounts of the standard equivalent to 100, 200, 400, 600, 800 and 1000 micromicrograms of the biotin standard; add water to 10 cc. if necessary and determine the pH of the tubes after 24 hours' incubation as above. Plot the pH reading against the corresponding amount of biotin in that tube on cross-section paper.

4. In assaying *blood*, use 1 cc. of a 1:10 dilution of citrated blood.

5. In assaying *urine*, use 1 cc. of a 1:100 dilution of urine. Interpolate from the curve the amount of biotin present in the sample.

**Note.**—Values between 0.07 and 0.10 microgm. per cent in blood and 3.0 to 3.1 microgm. per cent in urine have been found.

## METHOD FOR DETERMINATION OF NICOTINIC ACID IN BLOOD

**Principle.**—In this method, adapted after that of Klein, Perlzweig and Handler (*Jour. Biol. Chem.*, 145: 27, 1942), the color produced in the hydrolyzed tungstic acid blood filtrate by the reaction between nicotinic acid, cyanogen bromide and "Elon" (paramethylamino-phenol sulfate) is measured in the photoelectric colorimeter using color filter No. 42.

**Reagents.**—*Sulfuric acid solution*,  $\frac{2}{3}$  N.

*Sodium tungstate solution*, 10 per cent.

*Sodium hydroxide solution*, 40 per cent.

*Hydrochloric acid*, concentrated.

*Potassium dihydration phosphate solution*, 0.4M.—54.5 gm. of the anhydrous salt in 1 liter of water.

**Cyanogen Bromide Solution, 4 Per Cent.**—A cold, saturated bromine-water solution is carefully decolorized in the cold with freshly prepared 10 per cent sodium cyanide measured from a buret. This solution, in a dark colored bottle kept in the cold, is stable for long periods of time. A portion is adjusted to pH 4.5 before use.

**Elon.**—Solid (Eastman).

**Stock Standard Nicotinic Acid Solution.**—Dissolve 250 mg. of nicotinic acid in about 50 cc. of alcohol, transfer to a 100 cc. volumetric flask and dilute to the mark with alcohol (1 cc. = 2.5 mg.).

**Working Nicotinic Acid Standard.**—Dilute 1 cc. of the stock to 500 cc. with water just before use (1 cc. = 0.005 mg.).

**Procedure.**—1. A 1:5 tungstic acid filtrate is prepared from 5 cc. of blood, 10 cc. of water, 5 cc. of 10 per cent sodium tungstate and 5 cc. of  $\frac{2}{3}$  N sulfuric acid. The precipitate is separated by centrifugation and the supernatant freed from any small particles by recentrifugation.

2. Place 15 cc. of the clear supernatant (equivalent to 3 cc. of blood) in a tube graduated at 15 cc.; add 2 cc. of concentrated hydrochloric acid and heat the uncovered tube in boiling water for 1 hour.

3. Cool and adjust the pH to about 4.5 by the addition of first, 40 per cent sodium hydroxide solution (about 1.8 cc.), then approximately N sodium hydroxide solution dropwise as the proper degree of acidity is approached. "Alkacid" paper may be used to estimate the pH.

4. Replace the tube in boiling water and reduce the volume, if necessary, to the 15 cc. mark. Cool and adjust to 15 cc. (If test tubes of about 23 mm. diameter have been used, the volume will have been sufficiently reduced so that this second heating will probably not be needed.)

5. Transfer 5 cc. portions of the solution to each of 2 test tubes.

6. Add 1 cc. (= 5 micrograms of nicotinic acid) of the diluted nicotinic acid standard to the 5 cc. remaining in the digestion tube and 1 cc. of water to each of the other 2 tubes.

7. Add to each tube 0.5 cc. of the 0.4M phosphate solution and heat in water at 80° C. for 5 minutes.

8. Add 0.5 cc. of the cyanogen bromide to the tube containing the standard and to one of the other 2 tubes. Add 0.5 cc. of water to the remaining tube; this is the blank. Continue heating all 3 tubes for 5 minutes longer.

9. Cool all tubes to room temperature with running tap water and add 150 mg. of Elon to each tube.

10. Mix thoroughly by inversion and allow to stand in the dark for 30 to 40 minutes.

11. Read in the colorimeter, using filter No. 42, and a distilled water zero.

**Calculation.**—Subtract the reading of the blank from the reading of the unknown, and also from the reading of the tube containing the unknown plus the added 5 micrograms of nicotinic acid. The difference in readings of the last two represents the colorimeter reading due to 5 micrograms of nicotinic acid, = D. Then  $\frac{5}{D} \times \text{reading of unknown (corrected for the blank)} = \text{micrograms of nicotinic acid per cc. of blood.}$

**Note.**—Normal fasting human blood contains 3 to 5 micrograms per cc.

### METHOD FOR DETERMINATION OF N<sup>1</sup>-METHYLNICOTINAMIDE IN URINE

**Principle.**—In this method, described by Huff and Perlzweig (*Jour. Biol. Chem.*, 150: 483, 1943), the fluorescence of the butanol extract of alkalized urine is measured in a suitable electric fluorophotometer or a fluorocomparator.\*

**Reagents.**—*Lloyd's reagent*

*Sodium hydroxide solution, 40 per cent*

*Normal butyl alcohol*

**Procedure.**—1. To 10 cc. of urine add 3 drops of 40 per cent sodium hydroxide and 0.5 gm. of Lloyd's reagent. Mix and centrifuge. The supernatant is used for the blank.

2. Place 5 cc. of the blank in a 125 cc. separatory funnel.

3. Place 5 cc. of the untreated urine in another 125 cc. separatory funnel.

4. Add to each, 5 cc. of n-butanol and 1 cc. of 40 per cent sodium hydroxide solution. Shake vigorously, allow the 2 layers to separate and draw off and discard the lower aqueous layer.

5. The upper layer is run into a centrifuge tube, the tube capped and centrifuged until the butanol is clear.

6. About 4 cc. of each are pipetted into standard tubes and examined for fluorescence in a fluorocomparator. Subtract the blank value.

**Notes.**—1. When the urine contains more than 3 micrograms per cc., dilute the urine to a suitable volume with 25 per cent potassium chloride and proceed as above on a 5 cc. aliquot.

2. It has been found that preliminary adsorption by permutit and elution with salt solution are unnecessary; the results by the simplified method are substantially similar.

3. The chief excretion product of nicotinic acid metabolism in human urine is N<sup>1</sup>-methylnicotinamide; the above determination is thus a more satisfactory guide than determinations of nicotinic acid.

4. The presence of fluorescence in urine obtained after the morning voiding, but before breakfast indicates that the individual is adequately supplied with nicotinic acid.

### METHOD FOR DETERMINATION OF PYRIDOXINE IN URINE

**Principle.**—In this method, adapted after that of Scudi (*Jour. Biol. Chem.*, 139: 707, 1941), the urine is freed of interfering substances by adsorption on superfiltrol. The pyridoxine is eluted and the blue color developed with a butanol solution of 2, 6-dichloroquinonechloroimide which is then measured in the photoelectric colorimeter using red filter No. 66.

**Reagents.**—*Superfiltrol*

*Normal butyl alcohol, acid-free, reagent grade*

*Veronal buffer (pH 7.6 to 7.8).*—Dissolve 15 gm. of sodium diethyl barbiturate in 700 cc. of water. Add dilute hydrochloric acid until the proper pH is attained

\* May be obtained from W. A. Taylor & Co., Baltimore, Md.

(bromthymol blue on spot plate or the glass electrode). Filter from the precipitated barbituric acid. Check the pH from time to time.

**Citrate buffer (pH 3.0 to 3.5).**—Dissolve 21 gm. of citric acid in water in a 1-liter volumetric flask. Add 200 cc. of carbonate-free N/1 sodium hydroxide and dilute to the mark with water. Place 45 cc. of this solution in a 100 cc. volumetric flask and dilute to the mark with N/10 hydrochloric acid.

**Chlorimide reagent.**—Dissolve 50 mg. of 2,6-dichloroquinonechloroimide (Eastman) in 1600 cc. of normal butanol. Store in a refrigerator in a brown bottle. Keeps about 2 weeks. Samples withdrawn for use are first allowed to come to room temperature.

**Stock Standard Pyridoxine Solution.**—Dissolve 100 mg. of pyridoxine hydrochloride (Merck) in water in a 100 cc. volumetric flask. Add 2 drops of concentrated hydrochloric acid and dilute to the mark with water. Store in a brown bottle in the refrigerator (1 cc. = 1 mg. pyridoxine hydrochloride).

**Working standard pyridoxine solution.**—Prepared fresh from the stock solution by diluting 5 cc. to the mark in a 1-liter volumetric flask (1 cc. = 5 microgm.).

**Procedure.**—1. Pipet 5 cc. of urine into a test tube, add 10 mg. of superfiltrol and 2 cc. of the citrate buffer.

2. Stopper the tube and shake vigorously several times during a 30-minute period.

3. Centrifuge the suspension and discard the clear supernatant.

4. Add 5 cc. more of the citrate buffer, shake, centrifuge and discard the supernatant.

5. In a second test tube place 1 cc. of the pyridoxine standard (= 5 microgm.).

6. Add to each tube 10 cc. of the chlorimide reagent and shake vigorously several times during the course of 5 minutes; then add 3 cc. of veronal buffer solution to each tube and agitate the mixture.

7. Centrifuge and pour the butanol water mixtures into separatory funnels.

8. Allow the layers to separate and draw off and discard the aqueous layer.

9. Add to each funnel about 1.0 gm. of anhydrous sodium sulfate, shake and allow the powder to settle. Pour off the clear liquid.

10. Read in the colorimeter approximately 20 minutes after the chlorimide reagent was added, using red filter No. 66 and a zero setting obtained from a tube of the chlorimide reagent.

**Calculation.**—
$$\frac{1.0}{\text{reading of the standard}} \quad (= \text{factor}) \times \text{reading of the unknown} =$$
  
pyridoxine hydrochloride in micrograms per cc.

**Notes.**—1. If more than 10 microgm. is to be expected use 5 cc. of a 1:5 dilution of urine, and multiply the result by 5.

2. Normal urine contains less than 1 microgm. per cc. Consequently, the detection of undernutrition is determined by the amount excreted in the urine 1 hour after the subcutaneous injection of 50 to 100 mg. of the vitamin. Normally more than 5 per cent of the injected vitamin is excreted. Values of less than 3 per cent are indicative of lack of dietary vitamin B<sub>6</sub>.

**METHOD FOR DETERMINATION OF ASCORBIC ACID IN BLOOD**

**Principle.**—In this volumetric method of Farmer and Abt (*Proc. Soc. Exper. Biol. and Med.*, 34: 146, 1936) the plasma proteins are precipitated with metaphosphoric acid and the ascorbic acid in solution titrated with standardized indophenol.

**Reagents.**—*2,6-dichlorophenol-indophenol-sodium solution.*—Dissolve 1 tablet (Hoffman-LaRoche) in recently boiled distilled water in a 100 cc. volumetric flask. These tablets have been standardized so that in the above solution 1 cc. will be reduced by 0.01 mg. of ascorbic acid. Keeps about 3 days in the cold, but is best made up fresh.

*Metaphosphoric acid solution, 5 per cent.*

**Procedure.**—1. Place 2 cc. of plasma in a 15 cc. centrifuge tube; add 4 cc. of water followed by 4 cc. of the metaphosphoric acid solution. Mix by tapping and centrifugalize.

2. Place 2 cc. of supernatant in a test tube.

3. Place 1 cc. of the 5 per cent metaphosphoric acid plus 1 cc. of water in another test tube (blank).

4. Add the indophenol solution from a 5 cc. microburet (graduated to 0.02 cc.) to each tube, dropwise, until a pink color is obtained that persists for 30 to 60 seconds.

**Calculation.**—The difference between the two titrations  $\times 0.01 =$  mg. of ascorbic acid in sample (0.4 cc. of plasma). Or  $\frac{\text{titration difference} \times 0.01 \times 100}{.4}$  or titration difference  $\times 2.5 =$  ascorbic acid per 100 cc. of plasma.

**Notes.**—1. The titrations should be carried out rapidly; they should be completed within 2 minutes.

2. Plasma ascorbic acid should be determined as soon as possible after taking blood sample.

3. Normal ascorbic acid blood content is 0.8 to 2.4 mg. per 100 cc. of plasma. Values below 1.1 mg. are found in clinical scurvy.

**SECOND METHOD FOR DETERMINATION OF ASCORBIC ACID IN BLOOD**

**Principle.**—In this colorimetric method of Mindlin and Butler (*Jour. Biol. Chem.*, 122: 673, 1938) the amount of color reduction of an indophenol solution acted on by blood plasma is measured and calculated in terms of ascorbic acid.

**Reagents.**—*Metaphosphoric acid solution, 5 per cent.* Keeps about 2 weeks.

*Potassium oxalate, 20 per cent.*

*Potassium cyanide, 5 per cent.*

**Acetate buffer.**—To 5 gm. of sodium acetate crystals, dissolved in 20 cc. of water, add 1 cc. of 1 per cent acetic acid. Transfer to a 100 cc. volumetric flask. Dilute to the mark with water.

**Dye Solution (Stock).**—Place 50 mg. of 2,6-dichlorophenolindophenol sodium in a 100 cc. volumetric flask and dilute to the mark with recently boiled, distilled water.

**Dye Solution (Dilute).**—Dilute 5 cc. of the stock dye solutions to 100 cc. with recently boiled, distilled water.



**Dye Acetate Solution.**—Mix equal parts of diluted dye solution and acetate buffer. Make up enough for 1 day's use only.

**Stock Standard Ascorbic Acid.**—Place 50 mg. of ascorbic acid in a 100 cc. volumetric flask. Add 50 cc. of the 5 per cent metaphosphoric acid solution, dissolve and dilute to the mark with water (1 cc. = 0.5 mg.).

**Dilute Standard Ascorbic Acid.**—Pipet 1.0 cc. of the stock standard into a 100 cc. volumetric flask, add 50 cc. of the 5 per cent metaphosphoric acid and dilute to the mark with distilled water (4 cc. = 0.02 mg.).

**Procedure.**—1. Make color comparisons in the Klett-Summerson colorimeter, using filter No. 54.

2. **Blank.**—Place 4 cc. of the indophenol-acetate solution into a colorimeter tube, add 4 cc. of 2.5 per cent metaphosphoric acid (made by diluting the 5 per cent solution with an equal volume of water); mix and read at once in the colorimeter against a distilled water zero.

3. **Factor.**—Place 4 cc. of the dilute ascorbic acid standard in a colorimeter tube, add 4 cc. of the dye-acetate solution and read at once in the colorimeter. Plasma ascorbic acid factor = 
$$\frac{2.0}{\text{Blank reading} - \text{standard reading}}$$

4. **Unknown.**—Place 1 drop each of 20 per cent potassium oxalate and 5 per cent cyanide solutions in a test tube. Add 4 to 5 cc. of freshly drawn blood. Mix and centrifuge. Place 2 cc. of the plasma in another test tube, add 2 cc. of water and 4 cc. of 5 per cent metaphosphoric acid. Mix by gentle shaking and filter.

5. Place 4 cc. of the dye acetate solution in a colorimeter tube. Add 4 cc. of the filtrate. Read at once in the colorimeter.

**Calculation.**—Blank reading — unknown reading  $\times$  factor = mg. of ascorbic acid per 100 cc. plasma.

**Notes.**—1. Readings should be made within 30 seconds.

2. Note that the procedure depends only on the *difference* in readings between blank and unknown and blank and standard. Thus changes in dye concentration will not affect the result as long as the same solution is used for both blank and unknown except that the dye concentration should not give readings that are too low. Dye blanks that read around 150 in the colorimeter are satisfactory. Because only the difference in readings is used, the dye blank need not be the same for the unknown as was used for the standard when the factor was obtained. It is essential, however, that the same dye concentration be used for both the blank and unknown in making this determination.

3. The standard ascorbic acid solution is only needed to standardize the dye solution. Once this is done and the factor obtained it is unnecessary to use a standard as long as the same conditions are maintained.

## METHOD FOR DETERMINATION OF ASCORBIC ACID IN URINE

**Principle.**—In this volumetric method of Harris and Ray (*Lancet*, 228: 71, 1935) the ascorbic acid is determined by titrating an acidified sample of fresh urine with a standard indophenol solution.

**Reagents.**—2, 6-dichlorophenol-indophenol-sodium solution.—Prepared as described on page 879.

*Acetic acid solution, 5 per cent.*—Add 95 cc. of water to 5 cc. of glacial acetic acid and mix.

**Procedure.**—Pipet 10 cc. of the indophenol solution into a porcelain dish. Add the acetic acid solution dropwise until a red color appears. Fill a 5 cc. microburet (graduated to 0.02 cc.) with freshly voided urine and add to the dye solution in the dish until the color disappears.

**Calculation.**—
$$\frac{10}{\text{cc. of urine used}} = \text{mg. ascorbic acid per 100 cc. of urine.}$$

*Note.*—Normal adult excretion is about 25 mg. per day.

## METHOD FOR DETERMINATION OF PARA-AMINO BENZOIC ACID IN BLOOD AND URINE

**Principle.**—This method, modified after that of Eckert (*Jour. Biol. Chem.* 148: 197, 1943), is based on that of Bratton and Marshall for sulfonamide compounds (pages 853 to 854).

**Reagents.**—*Trichloroacetic acid solution, 15 per cent.*

*Trichloroacetic acid solution, 2.7 per cent.*—Dilute 18 cc. of the 15 per cent solution to 100 cc. with water.

*Sodium nitrite solution, 0.2 per cent.*—Best to use freshly prepared but will keep a week in the refrigerator.

*Ammonium sulfamate solution, 2 per cent.*

*Hydrochloric acid solution, approximately 4N.*—Dilute 388 cc. of the concentrated acid to 1 liter.

**Color Reagent.**—Dissolve 0.1 gm. of N-(1-naphthyl) ethylenediamine dihydrochloride (Eastman or LaMotte) in 100 cc. of water.

**Stock Standard Para-aminobenzoic Acid.**—In a 500 cc. volumetric flask dissolve 100 mg. of the pure crystalline compound, dilute to the mark and mix (1 cc. = 0.2 mg.).

**Working Standard Para-aminobenzoic Acid.**—Pipet 1 cc. of the stock standard into a 100 cc. volumetric flask, dilute to the mark with 2.7 per cent trichloroacetic acid solution and mix (10 cc. = 0.02 mg.).

**Preparation of Filtrate.**—1. Place 30 cc. of water in a 50 cc. Erlenmeyer flask and add 2 cc. of oxalated or citrated blood. Shake and let stand 5 minutes.

2. Add 8 cc. of 15 per cent trichloroacetic acid, slowly with rotation of the flask.

3. Stopper the flask, shake vigorously and allow to stand for 15 minutes. Filter.

**Procedure for Free Para-aminobenzoic Acid.**—1. Place 10 cc. of the blood filtrate in a test tube and add 2 cc. of water.

2. Place 10 cc. of the working standard in a second test tube and add 2 cc. of water.

3. Place 10 cc. of the 2.7 per cent trichloroacetic acid in a third test tube and add 2 cc. of water.

4. To each tube add 1 cc. of 0.2 per cent sodium nitrite solution, mix and allow to stand 15 minutes.

5. To each tube add 1 cc. of 2 per cent ammonium sulfamate solution, mix and allow to stand 3 minutes.

6. To each tube add 1 cc. of the color reagent.

7. Allow to stand 30 minutes and read in the photoelectric colorimeter using a distilled water zero and filter No. 54.

**Calculation.**—Subtract the blank reading from the readings of unknown and standard when  $\frac{4}{\text{reading of standard}}$  (= factor)  $\times$  reading of unknown = mg. of para-aminobenzoic acid per 100 cc. of blood.

**Procedure for Total Para-aminobenzoic Acid.**—1. In a test tube graduated at 10 cc. place 10 cc. of the blood filtrate; add 0.5 cc. of the 4N hydrochloric acid and heat in boiling water for 1 hour.

2. Cool to room temperature and make up to the 10 cc. mark with water.

3. Add 2 cc. of water and proceed exactly as for the free acid above.

4. The calculation is the same.

The *conjugated para-aminobenzoic acid* is obtained by subtracting the free from the total.

**Procedure for Para-aminobenzoic Acid in Urine.**—The protein-free urine is diluted to contain about 2 mg. per cent and then diluted with an equal amount of 4N hydrochloric acid.

*Free para-aminobenzoic acid* is determined on a 10 cc. portion exactly as for blood.

*Total para-aminobenzoic acid* is determined as for blood, but is heated without the addition of any more acid.

**Notes.**—1. Since the above reaction is similar to that of the sulfonamide determination, it is evident these drugs must not be administered when para-aminobenzoic acid determinations are to be made.

2. Ingested para-aminobenzoic acid disappears rapidly from the blood stream; it is excreted mostly in the conjugated form.

## METHOD FOR DETERMINATION OF TOCOPHEROL IN BLOOD

**Principle.**—In the following method, adapted from that of Mayer and Sabotka (*Jour. Biol. Chem.* 143: 695, 1942), the tocopherol is extracted from the protein-free solution by absolute ether, freed from impurities by adsorption on and elution from floridin and reacted with iron-bipyridine to form a pink color which is measured in the photoelectric colorimeter with green filter No. 52.

**Reagents.**—*Formaldehyde, 37 per cent, neutral to phenolphthalein*

*Alcohol, 95 per cent and absolute*

*Ether, absolute; peroxide free*

*Benzene, C.P.*

*Cadmium sulfate-sulfuric acid.*—1 per cent cadmium sulfate in 1 per cent sulfuric acid.

*Potassium hydroxide solution, 2 per cent.*

*Sodium sulfate, anhydrous powder and 0.5 per cent solution*

*Floridin, 30/60 mesh activated at 480° C.*

**Color Reagent.**—250 mg. of ferric chloride and 500 mg. of alpha, alpha prime, bipyridine made up to 1 liter with glacial acetic acid.  
Stable for long periods.

**Standard Tocopherol Solution.**—A solution of tocopherol in absolute ethanol containing 10 mg. per 100 cc.

**Procedure.**—1. Place 10 cc. of serum in a 250 cc. separatory funnel; add 5 cc. of 2 per cent potassium hydroxide, 15 cc. of 37 per cent formaldehyde and 15 cc. of 95 per cent alcohol. Shake thoroughly.

2. Add 50 cc. of ether, shake, allow the layers to separate and remove the ether layer into another separatory funnel.

3. Extract the aqueous portion in the first separatory funnel twice more with 50 cc. portions of ether, adding the ether layers to the second separatory funnel.

4. Wash the combined ether layers twice with 15 cc. portions of the cadmium sulfate-sulfuric acid solution and then twice with 25 cc. portions of the 0.5 per cent sodium sulfate solution.

5. Shake up the washed ether solution with several grams of anhydrous sodium sulfate; let stand 1 hour and run the ether through a pledget of cotton in a funnel into an Erlenmeyer flask that has been filled with nitrogen.

6. Evaporate the ether by placing the flask in warm water.

7. Add 10 cc. of benzene; the residue should form a clear solution. If not, evaporate the benzene by immersing the flask in warm water and add another small portion of benzene. This process is repeated until a clear solution is formed with 5 c.c. of benzene.

8. Pass the clear yellow benzene solution through a column of flordin of 12 mm. diameter and 30 mm. height which has been filled with nitrogen.

9. Wash the column with 25 cc. of benzene, catching the colorless filtrate in a nitrogen filled 25 cc. volumetric flask.

10. Evaporate the benzene by immersing the flask in warm water. Redissolve the residue in 2.5 cc. of benzene.

11. Place 2.5 cc. of benzene in another 25 cc. volumetric flask and add 1 cc. of the tocopherol standard.

12. Place 2.5 cc. of benzene in a third 25 cc. volumetric flask.

13. Add 1 cc. of the color reagent to each of the three flasks and make up to the marks with absolute ethanol. Mix, allow to stand 20 minutes, and read the unknown and standard in the photoelectric colorimeter using color filter No. 52, with the blank (item 12) zero setting.

**Calculation.**—
$$\frac{1.0}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown}$$
  
= tocopherol, mg. per 100 cc. of serum.

**Notes.**—1. Both the benzene and the ether should not give a test with the color reagent.

2. The factor is valid over a wide range and a standard need not be run with each unknown.

3. Values ranging from 0.4 to 2.0 mg. per cent have been found in normal human serum.

## METHODS FOR TOXICOLOGICAL EXAMINATIONS

**Principles.**—1. It is highly important that a sufficient amount of material be furnished. Depending on the expected concentration of the substance, 50 to 1000 cc. of urine or 100 to 200 grams of feces are desired. In cases of acute poisoning with oliguria, small amounts of urine can be examined when more is not obtainable.

2. Material desirable for detection of some common poisons are as follows:

(a) *Lead*: Feces, blood and urine. Feces contain more than urine.

(b) *Mercury*: Urine, feces and stomach contents. Excretion about equal in urine and feces. Urine is desired when obtainable, as analysis is more quickly and easily done.

(c) *Arsenic*: Urine, blood, hair, some in feces.

(d) *Morphine*: Feces and urine.

(e) *Methyl alcohol*: Urine.

(f) *Carbon monoxide*: Blood.

(g) *Alkaloids, phenol, iodine, alkalis, etc.*: Vomitus and stomach washings.

(h) *Autopsy material*: Part of liver, brain, one kidney, stomach, bone and intestinal contents.

### REINSCH METHOD FOR THE DETECTION OF ARSENIC, ANTIMONY AND MERCURY

The Reinsch test possesses two advantages: (1) as a preliminary test when abundance of material is available and (2) for clinical purposes during the life of the patient. It is a test not only for arsenic, but for mercury and antimony as well. It may be applied directly to a liquid containing organic matter, as the urine, and may be completed in a few minutes. The Reinsch test can also be applied to tissue.

**Principle.**—If a solution containing an arsenite and acidulated with about one-fifth its volume of hydrochloric acid (arsenic free) is heated a little below the temperature of boiling water for  $\frac{1}{2}$  hour in the presence of metallic arsenic free copper foil (*keep copper below surface of liquid*), a gray stain is formed upon the copper which is an alloy of copper and arsenic. It is not formed in the presence of powerful oxidizing agents such as the chlorates. With an arsenate it is only slowly formed. If the presence of arsenates be suspected, it is well to reduce them to arsenites by sulphur dioxide and expel the excess of gas by boiling before applying the test. A stain having an appearance similar to that caused by arsenic is also formed if the liquid contains compounds of sulphur, selenium, gold, platinum, silver, bismuth, antimony or mercury.

To distinguish the arsenical stain from the others, the strip of thin copper foil, which should be about  $\frac{1}{8}$  by  $\frac{3}{4}$  of an inch, is taken from the solution, gently washed and dried by contact with filter paper. It is then inserted into one end of a clean piece of thin glass tubing, open at both ends and about 8 inches long. This is held at an angle of about 10 degrees to the horizontal and gently warmed along its entire length until the interior of the tube and the foil are perfectly dry. The portion of the tube immediately above the copper is then slightly warmed (to insure the formation of larger crystals than would be deposited upon a cold surface) and then, the forefinger being more or less applied to the upper opening in such a manner as to allow a very slow current of air to go through the tube, the copper is heated strongly. There is danger of loss if the air current is too rapid.

Of the compounds mentioned, selenium, arsenic, antimony and mercury are the only ones which produce a sublimate in the tube. Sulphur is volatilized as sulphur dioxide, and the other metals remain upon the copper.

The sublimate produced by mercury is grayish rather than pure white, and, when examined with the microscope the mercury deposit when rubbed, assumes a silvery appearance. Arsenic, antimony and bismuth give a gray or black deposit, is found to consist of an aggregation of shining globules. The deposits of the oxides of arsenic, antimony and selenium are white and more closely resemble each other, but differ in certain particulars. The *antimonial deposit* is nearer to the point at which heat was applied than the arsenical, and a portion of it may be in that part of the tube which was in the flame. After the formation of the sublimate it may be readily driven along the tube by a moderate heating if it be *arsenic*, while much higher temperature is required to volatilize the antimonial deposit. The arsenical deposit consists entirely of brilliant octahedral crystals varying in size, the larger being in the portion of the sublimate nearest to where heat was applied. The crystals are bright, with finely defined edges, and scintillate when the tube is rotated on its axis in the sunlight. The *antimonial deposit* is generally entirely amorphous. It may, however, contain crystals some of which may be octahedral of the same shape as the arsenical crystals, but rather duller in luster and less transparent, and whose edges appear as broader black lines. These crystals, if present at all, are always few in number and are surrounded by much granular material, and require a high temperature for their volatilization. Occasionally prismatic crystals are also formed either beyond the copper or in that part of the tube which was in the flame.

The presence of *selenium* is exceptional, originating most frequently as an impurity of sulphuric acid. Microscopically its sublimate is found to consist of amorphous material and may contain prismatic crystals arranged in feathery bundles.

Two points are to be borne in mind: Hydrochloric acid is rarely free from arsenic and the copper foil may contain it. The method should, therefore, never be used without a blank. If the chemicals be pure the copper is, if anything, brightened. Should it become dimmed in the slightest degree, the acid, which is usually at fault, must be rejected. An objection to this method is the fact that copper is introduced into the articles under examination. It should therefore never be used except with a small sample of the available material.

The practical limit of delicacy of this test is about 0.0065 milligram. It is certainly inferior in delicacy to the Marsh test.

It should also be remembered that normal urine may and frequently does, contain arsenic. The amount of this so-called normal arsenic is variable, depending upon food, occupation and environment. The Reinsch test is hardly sufficiently delicate to detect this "normal" arsenic, but both the Marsh test and the Gutzeit test usually will give a test for arsenic with normal urine.

In the case of tissue, 5 to 10 grams of finely divided material are diluted with distilled water. The mixture is then acidulated with concentrated hydrochloric acid and the Reinsch test conducted in the same manner as described.

**METHOD FOR THE DETECTION OF METALLIC POISONS**

**Oxidation of Organic Matter (Fresenius von Babo's Method).—**1 A portion of finely divided material is mixed with distilled water to a fluid mass and placed in a Kjeldahl flask.

2. About 30 cc. of concentrated hydrochloric acid (arsenic-free if a test is to be made for arsenic) are added per 100 cc. of material (a large excess of hydrochloric acid should be avoided).

3. Add 1 to 2 grams of potassium chlorate, shake well and set the flask upon a boiling water bath under the hood. Nascent chlorine is evolved which destroys the organic matter. When the flask is hot, it is frequently shaken and a small amount of potassium chlorate (0.3 to 0.5 gram) is added from time to time until the solution is clear or turbid, has a pale yellow color, and additional heating produces no further change. Fat is very resistant to oxidation by chlorine.

4. When oxidation is complete, dilute with hot water and add a few drops of dilute sulphuric acid to precipitate possible barium; shake and pour the liquid through a wet filter paper.

5. Evaporate in a porcelain dish on a water bath nearly to dryness to remove excess acid. The decomposition of some potassium chlorate may give a brown color at this point. (If necessary, filter, wash with water, and evaporate again almost to dryness.)

6. Dissolve in water and filter. (The insoluble residues may contain silver chloride, barium sulphate and lead sulphate in addition to fat. These can be identified if necessary, after fusion with potassium nitrate and sodium carbonate.)

Nitric and sulphuric acids with a trace of potassium permanganate may be used as a substitute for oxidizing purposes.

**Treatment with Hydrogen Sulphide.**—The filtrate should have only a faint yellow color, and should be slightly acid (test with litmus). Place in a flask and heat on a water bath. While heating saturate the solution with washed hydrogen sulphide from a Kipp generator. (If a test is to be made for arsenic, the hydrogen sulphide must be arsenic free. Prepare arsenic-free hydrogen sulphide by saturating dilute sodium hydroxide solution with hydrogen sulphide from crude iron sulphide and commercial hydrochloric acid.) Pour this sodium hydrosulphide ( $\text{NaSH}$ ) solution into a separating funnel and add slowly to dilute (1:4) sulphuric acid. Pass hydrogen sulphide into the hot solution for 30 minutes and continue for about 30 minutes after the flask has cooled, then stopper tightly and let stand for several hours, preferably overnight, and filter. The filtrate may contain chromium or zinc. The precipitate may contain arsenic, antimony, tin, mercury, lead, bismuth, copper, cadmium. Treatment with hydrogen sulphide almost invariably causes a precipitate of sulphuric and organic thio-compounds; therefore no positive conclusion can be drawn from the formation of precipitate at this stage.

**Examination of the Precipitate.**—The precipitate is thoroughly washed with hydrogen sulphide water and while still moist about 5 to 10 cc. of a boiling solution of equal parts of ammonium hydroxide and yellow ammonium sulphide are dropped upon the precipitate on the filter. Repeat this several times. Finally wash with a few cc. of a fresh mixture of ammonia and yellow ammonium sulphide. The filtrate may contain arsenic, antimony, tin, and copper (see Metallic Poisons I). The precipitate

may contain mercury, lead, copper, bismuth, and cadmium (see Metallic Poisons II).

**Metallic Poisons I.**—Evaporate the solution (the solution is often dark brown owing to dissolved organic substances) to dryness in a porcelain dish on a water bath, cool, moisten with fuming or concentrated nitric acid and again evaporate to dryness. Then mix the residue with three times its volume of a mixture containing 2 parts sodium nitrate and 1 part sodium carbonate. Thoroughly dry this mixture upon the water bath and introduce small portions at a time into a porcelain crucible containing a little fused sodium nitrate heated to redness. After the final addition heat the crucible a short time, introducing possibly a little more sodium nitrate until the fused mass is colorless. In the presence of copper the melt is gray or black from copper oxide. Sodium arsenate, sodium pyro-antimonate, sodium stannate as well as stannic oxide and copper oxide may be present. Soften the cold melt with hot water and wash into a flask. Add a little sodium bicarbonate to decompose the small quantity of sodium stannate possibly in solution and precipitate all the tin as stannic oxide and then filter. The filtrate contains any arsenic present as sodium arsenate and the residue may contain sodium pyro-antimonate, stannic and copper oxide.

**Marsh Test for Arsenic.**—Acidify the filtrate with arsenic-free sulphuric acid. Evaporate in a casserole over a free flame, and add sufficient arsenic-free sulphuric acid to expel nitric acid. Heat until copious white fumes of sulphuric acid appear. Arsenic if present is in the form of arsenic acid and is tested in the Marsh apparatus (Fig. 334).

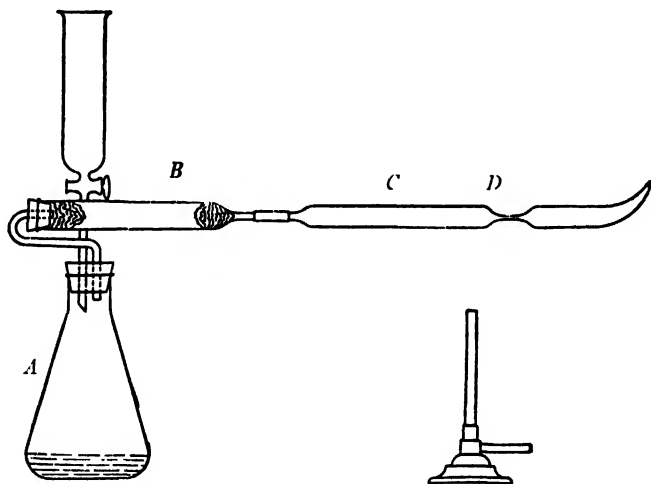


FIG. 334.—ONE FORM OF MARSH APPARATUS USED IN TESTING FOR ARSENIC

Place about 30 grams of arsenic-free granulated zinc in flask *A*. *B* should be packed with anhydrous calcium chloride. Since pure acid invariably will not react with pure zinc it is necessary to add about 100 milligrams of copper sulphate, thereby forming an electric couple and causing hydrogen to be formed as soon as acid is added. Stopper tightly and pour 50 cc. cold, dilute, arsenic-free sulphuric acid (1 volume concentrated sulphuric acid: 5 volumes water) into the funnel and regulate the flow of this acid upon the metal so that the hydrogen will not be generated too violently. The flask should be kept cool during the analysis by keeping it surrounded with cool water.



If the temperature gets too high, sulphur dioxide is formed and this in the presence of hydrogen is reduced to hydrogen sulphide ( $\text{H}_2\text{S}$ ), which interferes with the test. All joints of the apparatus should be tight to avoid escape of arseniureted hydrogen ( $\text{AsH}_3$ ), commonly called "Arsine" and also to prevent explosions. Air should be completely expelled before igniting to prevent explosion. To tell when this point is reached, collect hydrogen in a dry test tube until it ignites without detonation when carried to a flame. It may require as long as 30 minutes to expel the air. Test the hydrogen to insure its entire freedom from arsenic. Neither the arsenic mirror nor spot appear. With the hydrogen burning at the outlet, gradually introduce the perfectly cold sulphuric acid solution containing arsenic in small portions into the flask *A* by means of the same funnel. At the same time heat ignition tube *C* to redness just back of the constriction *D*. Keep reaction mixture cool by surrounding with cold water. Heat promotes the formation of hydrogen sulphide by reduction of sulphuric acid. Hydrogen sulphide is in turn decomposed on ignition to sulphur which may lead to the formation of a "false mirror." Also, if the stream of gas is too rapid, arsenic may be lost. If the solution contains arsenic, a shining metallic arsenic mirror is deposited just beyond the point of ignition. Antimony will give a mirror before and beyond the point of ignition. If organic substances are present a carbon mirror may be formed, and selenium will produce a yellowish-red or brownish-red mirror. If the flame is removed from *C* and a cold porcelain dish pressed down on the arsenic hydrogen flame at the tip, a brownish black spot is formed upon the dish. This spot dissolves readily in sodium hypochlorite solution (Dakin's). Antimony spots will not dissolve.

Extinguish the flame at the end of tube *C* and hold in the escaping gas a strip of filter paper moistened with concentrated silver nitrate solution (1:1). A yellow stain appears if the hydrogen contains arsenic and a drop of water. (See note regarding presence of "normal arsenic" under Reinsch test.)

**Metallic Poisons II.**—Over the substance on the filter paper remaining after the treatment with the hot ammonium sulphide mixture there are poured several small amounts of warm, rather dilute nitric acid (1 volume concentrated nitric acid: 2 volumes water). Mercuric sulphide does not dissolve but all the other sulphides pass into solutions as nitrates. The filtrate may contain lead, copper, bismuth, and cadmium, and specific tests are available for their recognition. (If the filtrate contains lead, dilute sulphuric acid produces a white precipitate of lead sulphate.)

**TEST FOR MERCURY.**—Treat the substance remaining on the filter paper after nitric acid treatment, even though not black, with a little hot dilute hydrochloric acid containing a crystal of potassium chlorate, and pass the acid through the paper several times. Evaporate the filtrate to dryness in a porcelain dish upon the water bath and dissolve in 5 cc. of 5 per cent hydrochloric acid, filter and test the filtrate for mercury as follows:

(a) *Stannous Chloride Test.*—Add to a portion of the filtrate a few drops of stannous chloride solution. A white precipitate of mercurous chloride appears if mercury is present. Excess of stannous chloride, especially if heat is applied, reduces this precipitate to gray metallic mercury.

(b) *Copper Test.*—Put a few copper slugs (previously cleaned in concentrated nitric acid and washed) into a portion of filtrate. Mercury deposits as a gray spot on standing or on heating. Wash the slugs successively in water, alcohol, and ether. Dry thoroughly and heat in a small bulb-tube of hard glass. Mercury sublimes and collects

in small metallic globules on the cold sides of the tube. A trace of iodine vapor introduced into the tube immediately transforms the gray sublimate into scarlet mercuric iodide.

The following outline shows the general plan of the above system of analysis.

<b>Material:</b> Treated with hydrochloric acid and potassium chlorate. Dilute sulphuric acid. Filter.			
<b>Filtrate:</b> Saturate with warm hydrogen sulphide.			<b>Precipitate tested for silver, lead, barium.</b>
<b>Precipitate:</b> Treated with hot ammonium sulphide and ammonium hydroxide.		<b>Filtrate tested for chromium, zinc.</b>	
<b>Filtrate tested for arsenic, antimony, tin, copper. (Metallic Poisons I.)</b>	<b>Precipitate tested for mercury, lead, bismuth, copper, cadmium. (Metallic Poisons II.)</b>		

## METHOD FOR THE DETECTION OF LEAD

**Preliminary Treatment: Feces and Tissues.**—1. Free from water by heating in a porcelain dish (Coors).

2. After the material begins to char, bring to a dull red heat and ash. Ashing must be very carefully conducted at a temperature well below full red heat, otherwise part or all of the lead will be lost by volatilization. Fecal material usually ashes readily, but the tissues form a residue which must be repeatedly extracted before the entire char is consumed. Usually the material requires re-ashing as a certain quantity of inorganic salts fuse and prevent complete oxidation.

Dissolve in hydrochloric acid diluted with an equal part of water and boil for about 20 minutes. Dilute with distilled water and filter while hot.

3. After the first ashing the material should be cooled. It is essential that *all* the ash be dissolved, for frequently lead phosphate is present as an insoluble residue which may be mistaken for silica. If this residue is insoluble in hydrochloric acid it should be treated with a mixture of hydrochloric and tartaric acids (which dissolves lead phosphate), until the ash is quantitatively dissolved. (Tartaric acid even of good quality usually contains lead and, therefore, should be tested with hydrogen sulphide).

**Urine.**—1. Ammonium hydroxide is added to urine until it is strongly ammoniacal. This mixture is allowed to stand for an hour. In this reaction the earthy phosphates are precipitated and lead phosphate is carried down.

2. The gelatinous mass of phosphates settles into a compact mass from which the clear lead-free liquor may be decanted and the remainder rapidly filtered by suction on a Buchner funnel.

3. The filter paper containing the precipitate is ashed and the precipitate completely dissolved in dilute hydrochloric acid with the aid of heat if necessary. (The urine must be either freshly collected or well preserved with thymol, because crystalline phosphates which form when urine is allowed to become ammoniacal on standing,

do not completely remove the lead. Heating ammoniacal urine to increase the rate of settling of phosphates prevents complete recovery of lead.)

**Procedure.**—1. Carefully neutralize the hydrochloric acid solution with dilute sodium hydroxide using methyl orange as indicator. Add dilute hydrochloric acid until the solution is just acid to methyl orange.

2. Saturate the cold solution with washed hydrogen sulphide. If sulphides precipitate to any great extent during this process, they may be separated at once, but if no precipitate appears, the solution, saturated with hydrogen sulphide, should be allowed to stand overnight. Centrifuge and wash with boiled distilled water, three times altogether. (FeS is easily oxidized by the air to soluble  $\text{FeSO}_4$ .)

3. Dissolve the precipitate in nitric acid (3 to 5 cc. concentrated). Boil to expel hydrogen sulphide, cool, and neutralize with dilute sodium hydroxide, using phenolphthalein as indicator.

4. Acidify with dilute acetic acid, and add an excess of potassium chromate—2 or 3 drops of a saturated solution. If the solution is held against a dark background during this process a slight turbidity may be observed around the drop of added chromate in the presence of even very minute quantities of lead. To hasten the reaction the solution should be boiled for a few minutes. If no turbidity is apparent the solution should be allowed to stand overnight. A yellow precipitate indicates the presence of lead.

5. If quantitative estimation is desired, proceed as follows: Filter; wash with warm water to remove all soluble chromate from the filter paper, and wash the precipitate completely into an Erlenmeyer flask. Wash the filter paper with 2 to 5 cc. of 1:1 solution of hydrochloric acid followed by warm water, and collect in the same flask. The precipitate dissolves readily in hydrochloric acid. Add an excess of potassium iodide solution at once and titrate the liberated iodine with N/200 sodium thiosulphate, a drop or two of starch being added near the end-point as indicator:

1 cc. N/200 sodium thiosulphate = 0.345 milligram lead.

When only small amounts of lead (less than 1 milligram) are present, use a microburet graduated in 0.02 cc. The sodium thiosulphate should be made up and preserved with suitable precautions for prevention of decomposition by carbon dioxide. Re-standardize once a week.

### METHOD FOR THE DETECTION OF SMALL AMOUNTS OF LEAD

**Principle.**—The organic matter is destroyed by wet digestion, the lead removed as the dithizone complex in chloroform from alkaline solution and then from the chloroform with acidulated water. The resulting red color is measured in the photoelectric colorimeter using green filter Nos. 51 or 52.

**Reagents.**—*Concentrated hydrochloric acid saturated with sodium chloride*

*Concentrated and 1 per cent nitric acid*

*Concentrated sulfuric acid*

*Glacial acetic acid*

*Perchloric acid, 72 per cent*

*Concentrated ammonium hydroxide:* Distill C.P. ammonia in an all glass (pyrex) still. Keep in a glass stoppered pyrex bottle.

*Citric acid solution:* Dissolve 4 gm. of the monohydrated acid in 100 cc. of 0.1 per cent salicylic acid.

*Calcium chloride, 10 per cent*

*Ammonium oxalate, C.P.*

*Chloroform, redistilled*

*Potassium cyanide, 10 per cent*

*Dithizone solution (stock):* Dissolve about 100 mg. of the commercial reagent (Eastman) in 20 to 25 cc. of chloroform in a 125 cc. separatory funnel and shake out 4 times with 15 cc. portions of ammonium hydroxide solution (made by diluting the concentrated reagent with 99 parts of water). The dithizone passes into the aqueous phase. Draw off and discard the chloroform layer and filter the aqueous extract through a pledget of cotton inserted in a funnel into another separatory funnel. Acidify slightly (litmus) with dilute hydrochloric acid and extract the precipitated dithizone 3 times with 20 cc. portions of chloroform. Combine the chloroform extracts in a separatory funnel and wash twice with distilled water. Draw off the chloroform solution into a dark glass-stoppered bottle and add chloroform sufficient to make 100 cc. This solution will keep a long time in the refrigerator.

*Dithizone solution (dilute):* A sufficient quantity is prepared for use by diluting 1 volume of the stock with 29 volumes of chloroform.

*Stock standard lead solution.*—Dissolve 0.160 gm. of crystalline lead nitrate in a 1-liter volumetric flask; add about 10 cc. of concentrated nitric acid and dilute to the mark with water. This keeps a long time (1 cc. = 0.1 mg. lead).

*Working standard lead solution.*—Dilute 10 cc. of the stock to 100 cc. in a volumetric flask with 1 per cent nitric acid (1 cc. = 0.01 mg. lead). This weaker solution does not keep well because lead tends to separate out.

**Digestion of Blood.**—1. Measure 10 cc. of blood into a 300 cc. Kjeldahl flask, add 15 cc. of concentrated nitric acid and warm gently in a fume cupboard. If frothing becomes excessive remove from the flame and allow to cool. When the reaction has subsided resume heating until the volume is reduced to about 5 cc.

2. Add 2 cc. of concentrated sulfuric acid and continue heating until copious fumes are evolved.

3. Add 2 cc. of perchloric acid and heat for about 5 minutes after fumes appear. The final volume should be about 3 cc.

**Digestion of Urine.**—1. Place 100 cc. of urine in a glass-stoppered cylinder and adjust to approximately pH 4.5 with bromocresol green by the dropwise addition of glacial acetic acid.

2. Add about 0.5 powdered ammonium oxalate and mix.

3. Add 2 cc. of 10 per cent calcium chloride solution and shake thoroughly.

4. Transfer to 2 Folin digestion tubes, let stand 20 minutes, and then centrifuge. Discard the supernatant.

5. Dissolve the precipitate in 1 of the tubes with about 2 cc. of hydrochloric acid and transfer all of the solution, with the aid of 10 to 15 cc. of water, to the other tube.

6. Heat this tube over a microburner until the solution has a volume of about 2 cc.; add 2 cc. of perchloric acid and continue heating until the digest is colorless (about 10 minutes).

**Solution of the Digest.**—1. Dissolve the digestion residue (from either blood or urine) by the addition of 5 cc. of the sodium chloride-hydrochloric acid reagent, warming, if necessary, to bring any insoluble material into solution.

2. Drop a piece of litmus paper into the flask or tube and add ammonium hydroxide solution until just alkaline.

3. Add 5 cc. of the 4 per cent citric acid to the warm mixture. This should redissolve any precipitate occurring when the ammonia was added. Further warming will hasten solution.

**Isolation With Dithizone.**—1. Transfer the solution to a 125 cc. separatory funnel, add 1 drop of thymol blue indicator and 5 cc. of 10 per cent potassium cyanide followed by ammonium hydroxide dropwise until a pH of 8.5 is attained (blue-green to blue with thymol blue).

2. Immediately extract the lead by shaking with a 5 cc. portion of the dithizone working solution. Allow the layers to separate and note the color of the chloroform phase. The lead-dithizone complex is red; if excess of the green dithizone solution has been used the chloroform will be an intermediate color of purple or crimson. Observation of this color will furnish a clue to the progress of the extraction and dictate the amount of the next portion of dithizone solution required.

3. Draw off the chloroform layer into a small separatory funnel and continue the extraction with a 5 cc. (or smaller) portion of dithizone solution, adding this chloroform portion to the previous extract. Continue the extraction if necessary with smaller portions of dithizone until the green color of the added dithizone solution remains unchanged.

4. To the combined chloroform extracts add 20 cc. of 1 per cent nitric acid, shake, allow the layers to separate and run the chloroform into a second separatory funnel.

5. Run the acid solution into a 25 cc. graduated cylinder through a small pledget of cotton inserted in the stem of a funnel.

6. Wash the chloroform extract in the second separatory funnel with 5 cc. of 1 per cent nitric acid, discard the chloroform fraction and run the acid solution (which should be colorless) into the cylinder through the same funnel. Make to 25 cc. with 1 per cent nitric acid and read in the colorimeter against a water zero.

7. A "blank" using 10 cc. of distilled water, is carried through the entire procedure as for blood. Read in the colorimeter.

8. The lead from 1 cc. of the working lead standard is extracted with dithizone solution beginning with "Isolation with dithizone." Read in the colorimeter.

**Calculation.**—Subtract the reading of the "blank" from the unknown; then

$$\frac{0.1}{\text{reading of standard}} (= \text{factor}) \times \text{corrected reading of unknown} = \text{mg. of lead in blood per 100 cc. or mg. of lead in urine per liter.}$$

**Notes.**—1. The limiting factor in the determination of minute quantities of lead by the dithizone isolation and colorimetric procedure is probably the size of the reagent blank. With special care in the selection of the reagents and the use of clean glassware, the blank may be reduced to as low as 1 microgram. Most chemicals, with the possible exception of the cyanide, can now be purchased free of lead. The ammonia should, however, be distilled as indicated. All chemicals, including the distilled water, can be readily checked qualitatively for the presence of lead with dithizone and, to a certain extent, running through the blank and standard as indicated will serve to cancel out

small amounts of contamination. The blank and standard should be run in duplicate and need not thereafter be determined for each unknown unless the reagents are changed.

2. Another source of lead contamination is in the collection of the samples, especially the urine sample. The glass container for the urine sample should be lead-free and capped with a plastic cap. Gross errors are very likely to occur unless such precautions are taken.

3. The blood of normal individuals may show as much as 0.06 mg. of lead per 100 cc. and the urine up to 0.08 mg. of lead per liter.

4. In workers in certain industries with abnormal lead exposure as much as 0.07 mg. per cent may be found in the blood and 0.10 mg. per liter in the urine without showing clinical plumbism.

### MAREN METHOD FOR THE DETECTION OF MERCURY

**Principle.**—In this method (*Jour. Lab. and Clin. Med.* 28: 1511, 1943) organic matter is destroyed by wet digestion, the mercury extracted as the yellow dithizone complex, and the color measured in the photoelectric colorimeter.

**Reagents.**—*Sulfuric acid, concentrated*

*Nitric acid, concentrated*

*Hydroxylamine hydrochloride solution, 20 per cent*

*Hydrochloric acid, approximately 0.5N*

*Dithizone solution: 10 mg. per liter of chloroform*

**Mercury standard.**—In a 1-liter volumetric flask dissolve 0.1353 gm. of reagent brand mercuric chloride in 500 cc. of 0.5N hydrochloric acid, dilute to the mark with water (1 cc. = 0.1 mg. of mercury).

**Working mercury standard.**—Transfer 4 cc. of the mercury standard to a 1-liter volumetric flask, add about 500 cc. of 0.5N hydrochloric acid and dilute to the mark with water (25 cc. = 10 micrograms of mercury).

**Procedure.**—1. Place about 5 gm. or 5 cc. of the material, accurately weighed or measured, into a 300 cc. long-necked Kjeldahl flask fitted with a reflux air condenser about 20 inches long.

2. Add 5 cc. of concentrated sulfuric acid and 15 cc. of concentrated nitric acid and warm gently.

3. After the initial foaming has subsided, heat strongly until nitric acid fumes have ceased to be evolved and fumes of  $\text{SO}_3$  appear. If charring occurs before this, cool the digest somewhat and add 5 to 10 cc. more of nitric acid and resume heating.

4. Cool the digest somewhat, add 12 to 13 cc. of 0.5N hydrochloric acid and transfer quantitatively to a separatory funnel with the aid of about 10 cc. of water.

5. Add 2 cc. of the hydroxylamine solution.

6. In another separatory funnel place 25 cc. of the working mercury standard.

7. Add to each, 10 cc. of the dithizone reagent and shake each thoroughly to extract the mercury from the aqueous phase.

8. Let stand until the layers separate, then run off the chloroform solutions through pledgets of cotton, inserted in ordinary funnels, into colorimeter tubes.

9. Using color filter No. 49 and a zero setting obtained from the dithizone solution, read the colors of unknown and standard.

**Calculation.**—
$$\frac{0.01}{\text{reading of standard}} (= \text{factor}) \times \text{reading of unknown} = \text{mg. of mercury in the sample.}$$
 Divide this result by the amount of sample taken and multiply by 100 for mg. per cent of mercury.

*Notes.*—1. The amount of dithizone used is sufficient for about 25 micrograms of mercury; if more than this amount is expected, use a smaller sample or dilute the digest to a definite volume with 0.25N hydrochloric acid and use a 25 cc. aliquot.

2. The mercury dithizone is sensitive to light; therefore, during the extraction avoid direct sunlight.

3. The method cannot be used if the noble metals are present.

### GUTZEIT METHOD FOR THE DETECTION OF ARSENIC

**Principle.**—In this method the organic matter is destroyed by wet digestion and the arsenic estimated by the effect of the arsine generated from it on strips of mercuric bromide paper as compared to strips which have been acted on by arsine from known amounts of arsenic.

**Reagents.**—*Concentrated sulfuric acid*

*Concentrated nitric acid*

*Concentrated hydrochloric acid*

*Hydrogen peroxide, 30 per cent*

*Sodium sulfate, anhydrous powder*

*Zinc metal, 20 or 30 mesh*

*Stannous chloride solution.*—Dissolve 40 gm. of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  in concentrated hydrochloric acid. Make up to 100 cc. with concentrated hydrochloric acid.

*Mercuric bromide solution, 5 per cent in alcohol*

*Potassium iodide solution, 15 per cent*

*Sand.*—30 mesh white sea sand that has been acid washed and ignited.

*Lead acetate solution, 10 per cent*

*Stock standard arsenic solution.*—Place 1.32 gm. of arsenious oxide in a 1-liter volumetric flask. Add 35 cc. of 20 per cent sodium hydroxide solution and shake until dissolved. Dilute to the mark with water (1 cc. = 1.0 mg. arsenic). Transfer 20 cc. of this solution to a 1-liter volumetric flask and dilute to the mark.

*Working standard arsenic solution.*—Transfer 50 cc. of the second arsenic dilution to a 1-liter volumetric flask and dilute to the mark (1 cc. = 0.001 mg. of arsenic).

*Mercuric bromide paper, sensitized.*—Use commercial arsenic paper strips that are exactly 2.5 mm. wide and about 12 cm. long. Soak the strips for about 1 hour in the mercuric bromide solution. Remove the strips from the solution and dry them by waving in air. Just before complete dryness, place them between clean sheets of paper and subject them to pressure long enough to take out bends or curls. Store in a dry dark place. Just before use cut off 1 cm. square from the end to be inserted.

**Arsine Generators.**—Mark 2 oz. wide-mouthed bottles of uniform design at 40 cc. on opposite sides of their circumference (A, Fig. 335) and fit each bottle by

means of a perforated rubber stopper (F) with a glass tube (B) 1 cm. in diameter and 6 to 7 cm. long with a constriction at one end to facilitate connection. Place a small wad of glass wool (C) in the constricted bottom end of each tube and 4 gm. of the sand (D), making sure that the same amount of sand is in each tube. Moisten the sand with the lead acetate solution, removing excess solution by light suction. Above tube B connect a narrow glass tube (E) of 2.6 to 2.7 mm. internal diameter and 10 to 12 cm. long.

**Preparation of Standard Arsenic Strips.**—1. Insert a sensitized paper strip into each of 5 of the narrow tubes (E) in such a position that about 1 cm. protrudes beyond the ends of the tubes. Bend this portion over the outside of the glass so that it forms a support for the paper.

2. Place in 5 generator bottles (A), 1, 5, 10, 20 and 30 cc. portions of the working arsenic standards equivalent to 0.001, 0.005, 0.010, 0.020 and 0.030 mg. of arsenic. Add to each bottle 5 cc. of concentrated hydrochloric acid and water sufficient to bring the total volume in each bottle to 40 cc.

3. Add to each bottle 1 gram sodium sulfate, 5 cc. of the potassium iodide solution and 4 drops of the stannous chloride solution. Mix and heat to 90° C. on a water bath for 5 minutes and then cool to room temperature under running tap water.

4. Place the stoppers (F) loosely in position in the bottle, tube E having previously been tightly connected.

5. Weigh out 5 gm. of the zinc on a clean filter paper which has been creased in such a manner as to make a trough.

6. Raise stopper F slightly above the mouth of the bottle and pour the zinc in as rapidly as possible. Immediately insert the stopper tightly and place the generator in water at 20° to 25° C. for 1½ hours. The water should completely cover the generator bottle and the temperature should be kept below 25° C. by the addition of small portions of ice if necessary.

7. Remove the strips of paper and measure the length of the stain on both sides in mm. Average the 2 dimensions and plot a graph on cross-section paper using average length of stain in mm. as ordinates and the corresponding mg. of arsenic as abscissa.

**Ashing of Unknown.**—1. Use a quantity of sample to contain not more than 0.025 mg. of arsenic.

2. Place the weighed or measured sample in a 300 cc. Kjeldahl flask and add 25 cc. of concentrated nitric acid, then cautiously 10 cc. of concentrated sulfuric acid.

3. Place each flask in a fume cupboard on a Chaddock burner covered by an asbestos ring having a 1-inch hole. Warm slightly and discontinue heating if foaming becomes excessive.

4. When the reaction has subsided, resume heating until the mixture begins to darken. The solution must not be permitted to char as arsenic will be lost.

5. Remove from the flame, cool slightly, add 25 cc. more of the nitric acid and resume heating until fumes of SO<sub>3</sub> begin to be evolved.

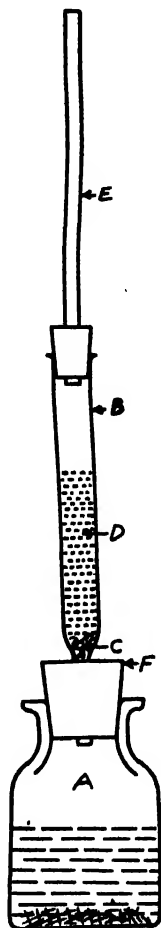


FIG. 335.—ARSENIC GENERATOR APPARATUS



6. Remove from the flame, allow to cool somewhat and cautiously add 5 drops of 30 per cent peroxide. Resume heating until  $\text{SO}_3$  fumes are again evolved.

7. If the solution is not now water clear or at most only a light yellow, repeat the addition of nitric acid, followed by the addition of peroxide. In any case the final heating to copious fuming should be made after the addition of the peroxide in order to eliminate the last traces of oxides of nitrogen.

8. When the organic matter has been completely oxidized, as indicated by the color of the solution, allow the contents of the flask to cool to room temperature and then wash down the sides of the flask with about 10 cc. of water.

9. Drop in a piece of litmus paper and cautiously add 40 per cent sodium hydroxide solution until just neutral; then add 5 cc. of concentrated hydrochloric acid.

10. Pour the contents of the flask into a generator bottle, rinsing the flask with as much water as is required to bring the final volume in the bottle to the 40 cc. mark.

11. Add the iodide and stannous chloride solutions as directed above for the standardization procedure; place a strip of sensitized bromide paper in position and proceed with the evolution of the arsine under exactly the same conditions as used in preparing the standard strips.

12. Measure and average the length of the stain on both sides of the strip and locating this length on the standard graph, read off on the abscissa the quantity of arsenic present in the sample.

*Notes.*—1. All the reagents, of course, must be arsenic free. Check this by running through the entire procedure with 1 gram of pure sugar as the sample.

2. Clean the sand in tube D, when necessary, without removing the sand from the tube by pouring through a little nitric acid followed by a water rinse and suction. Then once more moisten the sand with lead acetate solution.

3. Clean and dry tube E before each using. An ordinary pipe cleaner may be used.

4. Tube E should not be more than 1 or 2 mm. in internal diameter greater than the width of the paper strip; a wider tube permits the paper to curl, resulting in an uneven stain and a poor end point. Uneven staining may also result if the sensitized strip is more than 1 week old.

5. If more arsenic than 0.030 mg. is anticipated, wash the digest without previous neutralization into a 100 cc. cylinder, cool and dilute to the mark with water. Then pipet the proper aliquot into the generator bottle, neutralize with alkali, add the hydrochloric acid, dilute to 40 cc. and proceed with the arsine evolution as under procedure.

6. Since values as low as 0.001 mg. of arsenic are readily determined by this method the presence of arsenic will frequently be detected in normal urine. This is the so-called "normal" arsenic excretion.

### METHODS FOR THE DETECTION OF CARBON MONOXIDE IN THE BLOOD

**Tannic Acid Test.**—Prepare a solution of 1 part of blood with 4 parts of distilled water. Add 3 volumes of a 1 per cent solution of tannic acid solution and shake well. Normal blood is gray, but in the presence of carbon monoxide the blood gives a cherry-red (carmine red) color.

**Pyrogallic-Tannic Acid Test.**—1. Procure about 10 cc. of normal blood which has been prevented from clotting by the use of an anticoagulant (potassium or sodium citrate is the anticoagulant of choice) in the proportion of 0.05 gram per 10 cc. of blood.

2. Pipet 3 cc. of blood into a 50 cc. graduated cylinder and dilute with distilled water to the 30 cc. mark.

3. Saturate the rest of the blood with 3 to 5 per cent carbon monoxide gas ("illuminating gas" may be used) and then dilute 3 cc. to a volume of 30 cc. with distilled water.

4. From these solutions of oxyhemoglobin and carbon monoxide hemoglobin, mixtures are made which vary from 0 to 100 per cent in steps of 10 as follows:

Per Cent	Carbon Monoxide Hemoglobin, cc.	Oxyhemoglobin, cc.
100 .....	2	0
90 .....	4.5	0.5
80 .....	4	1
70 .....	3.5	1.5
60 .....	3	2
50 .....	1	1
40 .....	2	3
30 .....	1.5	3.5
20 .....	1	4
10 .....	0.5	4.5
0 .....	0	2

Two cc. amounts of these solutions are placed in small test tubes of uniform bore.

5. To each standard thus prepared are added 2 cc. of strictly fresh pyrogallic-tannic acid solution and the tubes are inverted twice to insure mixing. Do not shake.

6. The tubes should be sealed immediately by pouring a little melted paraffin on top of the contents while the tube is immersed in cold water as a precaution against overheating.

**Method of Sayers and Yant.**—Dilute 0.5 or 1 cc. of the whole blood to be analyzed, prevented from clotting by the addition of an anticoagulant, 1:10 with distilled water.

2. Pipet 2 cc. of this diluted blood into a test tube uniform in bore with those of the standards and add with a pipet 2 cc. of strictly fresh pyrogallic-tannic acid solution. Invert the tube several times to insure mixing. Do not shake!

#### REAGENT

Tannic acid, C.P. (2 per cent sol.)..... 25 cc.

Pyrogallic acid, C.P. (2 per cent sol.)..... 25 cc.

To be made up fresh.

3. Let stand 15 minutes and read against the standards. If carbon monoxide is present, let stand 15 minutes longer, read and report the latter reading as "per cent blood saturation with carbon monoxide."

**Notes.**—1. In carbon monoxide bloods prevented from clotting by oxalate, there is an appreciable change of carbon monoxide to carbon dioxide on standing. This is not true when citrate or sodium fluoride are used.

2. When saturating blood with carbon monoxide, in order to prevent loss of blood due to excessive foaming, the operation should be carried out in a beaker with constant stirring.

3. The blood for the standards should be saturated with carbon monoxide before it is diluted with distilled water in order to minimize the volume of carbon monoxide gas physically dissolved in the resulting solution.

4. If it is desired to preserve the standards, air must be excluded. The tube walls above the paraffin should be thoroughly dried and a permanent seal made by placing a disk of cardboard on top of the paraffin and filling the remainder of the tube with ordinary sealing wax. Standards thus prepared and kept in a cool place will retain their permanence for 1 to 2 weeks, not changing enough to interfere with the accuracy of the determination.

5. This method is not reliable for a CO blood content below about 10 per cent. For smaller amounts the succeeding method is recommended.

**Method of Roughton.**—*Principle.*—According to this method (*Jour. Biol. Chem.* 137: 617, 1941) the blood is laked and shaken in the Van Slyke-Neill closed type manometric apparatus with sodium glycinate-hyposulfite mixture which unites chemically with the  $O_2$  and  $CO_2$ , the liberated  $N_2$  is ejected and the pressure of the CO which is then evolved with ferricyanide is measured.

**Reagents.**—Saponin solution, 1 per cent.

*Glycinate-hyposulfite solution:* 3.75 gm. of glycine are dissolved in 45 cc. of carbonate-free 1N. sodium hydroxide and 1 gram of sodium hyposulfite added. Dissolve with a minimum of shaking and make up to 50 cc. To be used only on the day of preparation.

*Potassium ferricyanide solution*, 32 per cent.

*Octyl alcohol.*

**Procedure.**—1. Draw 4 drops of octyl alcohol into the reaction chamber of the Van Slyke-Neill apparatus.

2. Place 2 cc. of the saponin solution in the cup above the chamber.

3. Draw 2 cc. of the blood sample into the reaction chamber from a rubber tipped pipet placed against the stopcock so that the sample is drawn in before the saponin solution; then allow the saponin solution to flow in.

4. Close the upper stopcock and lake the blood by shaking for 1 minute.

5. Open the stopcock and draw in 1.5 cc. of the glycinate-hyposulfite mixture into the chamber which is then covered with black paper.

6. Close the upper stopcock, seal with a few drops of mercury, open the lower stopcock and evacuate the reaction chamber by lowering the mercury level to the 50 cc. mark.

7. Shake for 3 minutes, remove the black paper and carefully eject the liberated nitrogen by slowly raising the mercury level with the upper cock open until the liquid level rises to the bore of the stopcock.

8. Draw 0.5 cc. of the ferricyanide into the chamber, close and seal the upper stopcock with mercury and again evacuate the reaction chamber by lowering the mercury level to the 50 cc. mark.

9. Shake for 10 minutes, then measure the gas pressure at the 2.0 or 0.5 cc. mark, depending on the amount of CO present.

10. Eject the liberated gas carefully as above, then close the stopcock and read

the mercury column at the same volume (2 or 0.5 cc.) at which the gas pressure was determined.

11. Determine a blank correction with water in place of blood. It usually does not exceed 0.5 mm. of mercury.

12. Note the temperature.

**Calculation.**—The difference between the two pressure readings (9 and 10) minus the blank is multiplied by a constant to give the carbon monoxide in volume per cent. The constant varies with the temperature and may be obtained from the following table:

**FACTORS FOR CALCULATION OF CO CONTENT OF BLOOD**

Temperature °C.	Volume Per Cent CO	
	a = 0.5 cc.	a = 2.0 cc.
15	0.0317	0.1251
16	15	46
17	14	42
18	12	37
19	11	32
20	09	28
21	08	24
22	06	19
23	05	15
24	03	10
25	02	06
26	01	02
27	0.0299	0.1198
28	98	93
29	96	89
30	95	85
31	94	81
32	92	77
33	91	73
34	90	69

### METHOD FOR DETECTION OF METHEMOGLOBIN

**Principle.**—The method depends upon the fact that both hemoglobin and methemoglobin are changed quantitatively to cyanhemoglobin by dilute solutions of potassium cyanide. The color of the latter is a brilliant orange-red and is very suitable for colorimetric comparison. The change of methemoglobin to cyanhemoglobin is rapid even in the cold. Hemoglobin, however, changes slowly at room temperature, and this difficulty is avoided by converting all the hemoglobin present into methemoglobin by use of potassium ferricyanide and then converting the methemoglobin into cyanhemoglobin. The resulting solution of cyanhemoglobin is compared with a standard of known strength in a Duboscq colorimeter.

The total amount of hemoglobin plus methemoglobin having thus been determined colorimetrically, the hemoglobin content of the blood containing the 2 pigments (hemoglobin and methemoglobin) is determined separately from the oxygen capacity, employing the gasometric technic of Van Slyke. The hemoglobin determined by the oxygen capacity is subtracted from the hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is methemoglobin.

**Standard.**—1. The hemoglobin content (grams per 100 cc.) is determined gasometrically.

2. Then place 10 cc. of oxalated or defibrinated blood, which is known to contain no methemoglobin, in a 500 cc. flask.

3. Hemolyze by adding 300 cc. water.

4. Add 2.5 cc. of 3 per cent potassium ferricyanide solution and let stand 20 minutes.

5. Now add 25 cc. of 0.1 per cent potassium cyanide solution and dilute to 500 cc.

The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

Example of calculation: Strength of standard equals 15 grams hemoglobin per 100 cc. blood. Comparison of cyanhemoglobin in colorimeter: Standard 10, unknown 12. Unknown has 10/12 of 15 or 12.5 grams of total blood pigment per 100 cc. Gasometric determination of hemoglobin equals 10 grams per 100 cc. Therefore, sample has 12.5 less 10 or 2.5 grams of methemoglobin per 100 cc.

**Procedure.**—1. Two cc. of oxalated whole blood are placed in a 100 cc. flask and 50 cc. of water are added, which produces hemolysis in a few seconds.

2. Add 0.5 cc. of a M/10 (3 per cent) solution of potassium ferricyanide, and let stand 20 minutes.

3. Now add 5 cc. of a 0.1 per cent potassium cyanide solution. The change to cyanhemoglobin is immediate.

4. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin which is expressed as grams of "total hemoglobin pigment" per 100 cubic centimeters of blood.

5. Determine the oxygen capacity by the gasometric method of Van Slyke and multiply by 0.746 to obtain grams of hemoglobin per 100 cc.

**Calculation.**—Total hemoglobin pigment per 100 cc. minus hemoglobin per 100 cc. which can bind oxygen equals methemoglobin per 100 cc.

## METHODS FOR THE DETECTION OF CYANIDE

**Principle.**—The principles involved are clearly indicated under the various procedures outlined. Great care must be exercised in carrying them out because of the dangers to the analyst and secondly the interfering substances.

**Organs Best Suited for Analysis.**—If the poison has been taken by mouth, the stomach contents and brain should be analyzed. Analysis of the brain is necessary for the purpose of ruling out the possibility of the poison having been introduced into the stomach after death. If the poisoning resulted from inhalation, the lungs and brain must be examined. In cases of poisoning by inhalation, usually none, or only the very

faintest trace, is found in the stomach contents. This is of tremendous importance from the medicolegal aspect.

**Method of Isolation of Cyanide.**—The tissues are cooled by keeping them in an ice-box. Two hundred to 500 grams of tissue are ground up. Care should be taken to keep the tissue cold since hydrocyanic acid may volatilize if warm. If stomach contents are analyzed, usually one-fifth of the total volume of the contents is used. The ground-up tissue or the stomach contents are placed in a 1-liter flask and acidified with tartaric acid. The material is then distilled with steam, using a well cooled condenser the tip of which is bent to serve as an adapter and dipped into 5 cc. of 5 per cent sodium hydroxide solution in a receiving flask. The latter should be packed in ice. One hundred cc. of distillate are collected, which is ample to recover all the cyanide present, the following tests being employed.

**Qualitative Tests.**—1. *Schönbein's Test.*—Suspend a strip of filter paper, impregnated with guaiac and copper sulphate, over the material in a flask, the paper being held in place by the stopper. (Dip strip of filter paper into a freshly prepared alcoholic solution of guaiac 1:10; then let dry; when dry, moisten it with dilute (1:10,000) copper sulphate solution.) If color does not change, cyanide is absent and no further tests need be made. If a blue color results, cyanide *may* be present. The test is very sensitive but not specific; hydrochloric acid, nitric acid, chlorine, bromine, ozone, hydrogen peroxide, as well as some other substances also give a positive test. This test may be used as a preliminary one at the necropsy table. The following 2 tests must be employed since they are specific for cyanide:

2. *Prussian Blue Test.*—To 5 cc. of distillate, add 3 cc. of 25 per cent sodium hydroxide, then a few drops of freshly prepared ferrous sulphate solution and a few drops of ferric chloride solution. Warm a little. Let cool and add concentrated hydrochloric acid, drop-wise, until the dirty brown precipitate just dissolves; avoid excess hydrochloric acid. If cyanide is present, a deep blue precipitate (Prussian blue) appears. If only a trace of cyanide is present, a green solution results instead of a blue precipitate, but, on standing several hours, a small flocculent Prussian blue precipitate settles (sensitive to one part in 50,000).

3. *Liebig's Test.*—To 10 cc. of distillate, add 1 cc. of yellow ammonium sulphide and evaporate to dryness on the water bath. When dry, add 5 cc. of 5 per cent hydrochloric acid solution, warm a little and stir well to dissolve all of the thiocyanate that was formed during the evaporation. Let stand 2 hours, then filter. To the filtrate, add 5 to 10 drops of 10 per cent ferric chloride solution. If cyanide is present, a deep red color results. (This test is sensitive to 1 part in 10 million.)

The following tests may be used, but they are not specific for cyanide:

4. *Vortmann's Test.*—To 5 cc. of distillate, add a few drops of potassium nitrate solution, then 2 to 4 drops of ferric chloride solution and then enough dilute sulphuric acid until the color of the solution becomes a bright yellow. The solution should then be boiled, after which cool and add ammonium hydroxide until all of the iron is precipitated. Filter off the precipitate. To the filtrate add a few drops of a very dilute solution of ammonium sulphide. If cyanide is present, a play of colors results, violet, blue, green, yellow.

5. *Picric Acid Test.*—To 5 cc. of distillate (slightly alkaline) add a few drops of picric acid solution and warm gently. If cyanide is present, a red color develops (sensitive to 1 part in 1 million).

6. *Phenolphthalein Test*.—To 5 cc. of distillate, add a few drops of alkaline phenolphthalein solution (reduced phenolphthalein), then a few drops of 1:2000 copper sulphate solution. If cyanide is present, a red color develops (sensitive to 1 part in 20 million).

7. *Silver Test*.—To 2 cc. of distillate, add nitric acid until reaction is acid, then add a few drops of silver nitrate solution. If cyanide is present, a white precipitate of silver cyanide results.

**Quantitative Test**.—For quantitative analysis, a weighted amount of tissue is distilled, as described in the qualitative procedure. In the receiving flask, however, instead of having dilute sodium hydroxide, 20 cc. of 10 per cent silver nitrate solution are used, acidified with nitric acid. In order to make certain that all of the cyanide is isolated, distillation is continued until 200 cc. of distillate are obtained. During the distillation, the cyanide precipitates as silver cyanide. This is then filtered through a previously weighed Gooch crucible, washed, dried and weighed. From the weight of the silver cyanide, the amount of cyanide in the material analyzed is calculated as HCN.

In quantitative analysis, if the poison was taken by mouth, the entire amount of cyanide is determined in the gastro-intestinal tract. This is then multiplied by 100/98, which gives the amount in the entire body. This fraction is used because approximately 98 per cent of the cyanide, if taken by ingestion, remains within the stomach contents. If the poison was introduced by inhalation or injection, parts of all the organs and tissues are analyzed and, from these results, the amount present in the entire body is calculated.

The lethal dose of cyanide is accepted as 50 mgm., calculated as hydrocyanic acid.

The following factors interfere with the determination of the presence of cyanide:

1. Traces of hydrogen cyanide are produced during the first few days of putrefaction, but this disappears in the later stages.
2. Cyanides present in the tissues disappear during prolonged putrefaction and are changed to sulphocyanide.
3. In stomach contents, where the bulk of the cyanide remains at death, putrefaction is of little importance.
4. Embalming with formaldehyde interferes greatly in the tests, the cyanide forming condensation products with the formaldehyde.

It is, therefore, important that early toxicological analysis be made in cases of suspected cyanide poisoning. It is likewise necessary to rule out the presence of ferrocyanides, ferricyanides and thiocyanates before distillation is begun, because these compounds, when distilled in the presence of mineral acids, yield hydrogen cyanide.

Diffusion of the poison in a body after death has been recorded, but the process is extremely slow and it has been shown by Gettler, that, when cyanide is introduced into the stomach after death, no cyanide reaches the brain even after an interval of 2 months.

Although the term "cyanide of potassium" is generally employed in referring to cyanide poisoning, it is not the potassium salt, but because of its cheaper cost, the sodium salt that is used.

**METHOD FOR THE DETECTION OF BORIC ACID**

The specimen of urine, stomach washing or finely divided tissue is made strongly alkaline with a solution of sodium hydroxide or sodium carbonate and evaporated to dryness on the water bath. It is then carefully ashed at dull red heat. The ash is acidified with concentrated HCl, and divided into two portions. To one portion ethyl alcohol is added, and then ignited. A green flame indicates boron. This flame examined with the spectroscope gives four characteristic absorption bands in the yellow, green, and blue green. The second portion is diluted with a little water and tested with turmeric paper. If boric acid is present the paper turns a red brown, which is intensified on drying. In acute poisoning by mouth it is possible to determine whether boric acid or borax was taken, by analyzing the stomach contents or stomach washing. The internal organs and the urine are useless for this purpose. If the stomach washing is very alkaline it indicates borax; further, if the stomach contents are first dried on the water bath, ethyl alcohol will extract boric acid, but not borax. If it is desired to estimate the amount of boron present, the material made alkaline with  $\text{Na}_2\text{CO}_3$  is dried and ashed at low red heat. The ash is then thoroughly extracted with dilute, hot hydrochloric acid, transferred to a volumetric flask and water added to a definite volume. To 10 cc. of this solution is added some turmeric solution and the color that is produced is compared with that of a standard boric acid solution similarly colorized with turmeric solution.

**METHOD FOR THE DETECTION OF SODIUM CARBONATE**

The stomach contents, or the stomach washings (with water) must be used in analyzing for sodium carbonate (washing soda). Due to the presence of Na and  $\text{CO}_3$  ions in the blood and tissues normally, and also due to the buffer action of the blood and tissue fluids, it is impossible to definitely establish whether any exogenous sodium carbonate is present in the blood, tissues or urine. The stomach washings can be tested directly, unless a large amount of food is present. If so, the stomach contents should be dialyzed, and the resulting dialyzate used for the tests. A strip of litmus paper is introduced in order to ascertain whether the material is at all alkaline. If so, a few drops of phenolphthalein solution are added to some of the material. A deep red color is produced if sodium carbonate or sodium hydroxide is present. Sodium bicarbonate will give only a faint pink. To differentiate between sodium hydroxide and sodium carbonate, hydrochloric acid is added to some of the material. The liberation of much gas ( $\text{CO}_2$ ) indicates carbonate. For the quantitative estimation, a known excess of N/20 sulphuric acid is added to a measured amount of the diluted and filtered stomach contents, then boiled one to two minutes, cooled, and the sulphuric acid remaining is titrated with N/20 NaOH. From the amount of sulphuric acid neutralized by the stomach contents, the amount of  $\text{Na}_2\text{CO}_3$  is calculated.

**METHOD FOR THE DETECTION OF POTASSIUM CHLORATE**

The stomach contents, urine, blood, or finely divided tissues are placed into a dialyzing bag made of collodion or parchment paper. Dialysis into pure water is continued for several hours. To some of the dialysate is added an excess of silver nitrate



solution. The resulting precipitate of silver chloride is filtered off. The clear filtrate is acidified with nitric acid and sulphurous acid added, and the mixture is then brought to boiling. If a white precipitate remains it is silver chloride from the reduction of the chlorate by the sulphurous acid.

The simplest method for the quantitative estimation of the chlorate is to determine the chloride content of an aliquot portion of the material by one of the usual methods. A second measured portion of the material is made strongly alkaline with added sodium carbonate, well mixed, dried, and carefully ignited at low red heat. This procedure converts the chlorate into chloride. The chloride content is now determined in this ignited material. From the difference between the two chloride determinations, the amount of chlorate originally present can be calculated.

### **METHOD FOR THE DETECTION OF PHOSPHORUS**

In acute (white) phosphorus poisoning, the gastro-intestinal contents is of prime importance from an analytical standpoint. The material is distilled in a dark room screening off the light from the Bunsen burner. If white phosphorus is present, a luminescent ring will be noticed during the distillation, in the upper part of the water cooled condenser. For a further test, the distillate is strongly acidified with concentrated nitric acid and then evaporated to dryness on the water bath. This procedure converts the phosphorus to phosphoric acid, which can then be identified by the ammonium molybdate reaction.

### **METHOD FOR THE DETECTION OF ANILINE AND NITROBENZENE**

For the detection of aniline and nitrobenzene, the finely divided organs, urine, blood, or stomach contents are distilled with steam. If these substances are present in appreciable amount, the distillate is usually cloudy. To one portion of the distillate add two drops of chloroform and excess NaOH solution, and bring to boiling. If aniline is present, the very irritating and piercing odor of phenyl isocyanide develops. Should nitrobenzene be present, the steam distillate is shaken out with ether. The ether layer is allowed to evaporate spontaneously. If nitrobenzene was present, it will now be found in the form of a few oily globules with the characteristic odor of oil of bitter almonds. These drops are dissolved in alcohol. The alcoholic solution is reduced with powdered zinc and HCl. This converts the nitrobenzene to aniline. After making the solution alkaline with NaOH, the aniline obtained is tested by means of the isonitrile reaction as above.

### **METHOD FOR THE DETECTION OF CERTAIN ORGANIC VOLATILE POISONS**

For the detection of benzene, chloroform, ethyl-chloride, ether, carbon-bisulphide, carbon-tetrachloride and others of the many new volatile solvents and anesthetics considerable apparatus and technical skill are required which are hardly applicable in the average clinical laboratory. The methods are described in detail by Gettler and Siegel (Isolation from Human Tissues of Easily Volatile Organic Liquids and Their Identification, *Arch. Path.*, 1935, 19:208-212).

Nearly all these compounds have a characteristic odor which can be readily identified in the patient's breath or vomitus or in the stomach washings. Great care should be taken of vomitus or gastric washings to prevent the rapid evaporation of these poisons. The material must be placed in a tightly stoppered container and kept in the refrigerator. Frequently a simple distillation of the material on a water-bath, but using an electric plate for heating (to prevent ignition or explosion of some of these compounds) will bring out the characteristic odor. A long condenser, preferably cooled with ice-water, is desirable.

### **METHOD FOR THE DETECTION OF WOOD ALCOHOL (METHANOL)**

1. The material (vomitus, tissue, etc., or the contents of the suspected liquid) is acidified with tartaric acid and enough distilled water is added to make it fluid (to a volume of 100 cc.) and then steam distilled.

2. Collect 50 cc. of distillate, stopper and shake. Of this distillate 10 cc. is taken in a test tube and a heated copper spiral is plunged into the liquid. This is repeated 8 times and the test tube is held under cold running water. This converts the wood alcohol into formaldehyde. The test tube must be kept cold. Then 2 cc. of this oxidized liquid is poured into another test tube and the following test is made:

- (a) Add 10 drops of a 5 per cent sodium phenylhydrazine hydrochloride solution
- (b) Add one drop of a 0.5 per cent sodium nitroprusside solution
- (c) Add 3 drops of a 10 per cent sodium hydroxide solution

A blue color develops if formaldehyde is present—and indicates that wood alcohol (methanol) is present. The intensity of the blue color gives a rough estimate of the amount present.

A red color with the above reagents indicates acetaldehyde which is obtained in the manner described above when grain (ethyl) alcohol is present. For further tests see A. O. Gettler (Critical Study of Methods for the Detection of Methyl Alcohol, *J. Biol. Chem.*, 1920, 42, 2:311, 328).

### **METHOD FOR THE DETECTION OF GRAIN (ETHYL) ALCOHOL**

Much has been written concerning the detection of ethyl alcohol, especially in the presence of other alcohols, and also its medicolegal significance in intoxication. It has been fairly well established that the various methods to determine intoxication, such as an estimation of the amount found in the urine or expired air, are valueless, since they are excreted and no longer causing the inebriation. (Inebriation depends upon the amount of unoxidized alcohol in the brain.) Hence the ideal method is to determine the amount of alcohol present in the brain tissue. This of course is easy in the cases coming to necropsy, but correspondingly difficult in an intoxicated individual. In such cases examination of the spinal fluid is probably of greatest value. For further details the reader must consult the enormous literature which has accumulated.

### METHOD FOR THE QUALITATIVE DETERMINATION OF GRAIN (ETHYL) ALCOHOL

For an ordinary rapid method which is applicable to urine, blood, spinal fluid or finely divided tissue, the method of oxidation acid testing is carried out exactly as described under wood alcohol (methanol) and the red color obtained indicates the presence of ethyl alcohol. Large amounts of urine and blood are necessary for these determinations.

### METHOD FOR THE QUANTITATIVE DETERMINATION OF ETHYL IN TISSUES

**Principle.**—Depends upon the oxidation of ethyl alcohol to acetic acid and titrating with N/20 sodium hydroxide.

1. The tissue, as soon as it is removed from the body, should be placed in a tightly closed jar and placed in a refrigerator. When ice cold, 500 gms. are weighed out, quickly ground up and placed in a 2-liter flask. To this are added 600 cc. of water, 5 cc. of a saturated solution of tartaric acid and 1 cc. of white mineral oil. This mixture is now distilled with steam. A long, well cooled condenser should be used, and the distillation should be continued until exactly 800 cc. has been collected. The distillate is well mixed and used in the following procedure.

2. Twenty grams of potassium dichromate and 40 cc. of concentrated sulphuric acid are placed in a 500 cc. distilling flask. Three hundred cc. of the distillate obtained above is now added and the contents mixed well. The flask is then connected to a long, well cooled condenser by means of a Hopkins distilling head, and the distillation started. The heat must be so regulated that it will take from 45 to 50 minutes to collect exactly 250 cc. of distillate. After thoroughly mixing, 50 cc. of this distillate is titrated with twentieth normal sodium hydroxide solution, using phenolphthalein as an indicator. From this titration figure, the amount of alcohol present in 1 kilogram of brain tissue can easily be calculated as follows:  $(\text{cc. twentieth normal alkali} - 1.32) \times 71.58 = \text{mg. C}_2\text{H}_5\text{OH per kilogram of tissue}$ . The figure (—1.32) holds only for brain tissue. For other tissues a fair average would be calculated as follows:

$$1 \text{ cc. } \frac{N}{20} \text{ NaOH} = 2.3 \text{ mgms. of ethyl alcohol.}$$

The following rules have been laid down by Gettler and Tiber:

1. For the qualitative and quantitative determination of alcohol, the brain is of first importance.
2. The normal alcoholic content of human brain material is less than 0.0025 per cent.
3. The alcoholic content of the brain in persons who have partaken of alcoholic beverage ranges between 0.005 and 0.6 per cent.
4. All patients having an alcoholic content below 0.1 per cent show no abnormal physiologic effects.
5. Patients with an alcoholic content above 0.1 per cent and up to 0.25 per cent

show some physiologic disturbance, as evidenced by increased aggressiveness, and more or less loss of the sense of care. None of these patients, however, shows unbalanced equilibrium, which is commonly called intoxication.

6. When the alcoholic content rises above 0.25 per cent and up to 0.4 per cent and still higher to 0.6 per cent, the equilibrium of the person becomes unbalanced—a condition generally known as intoxication.

7. The degree to which any person is affected does not depend on the quantity of alcohol consumed, but on the amount of alcohol present in the brain at the time.

### CAVETT METHOD FOR THE DETERMINATION OF ETHYL ALCOHOL IN BLOOD, URINE AND OTHER BODY FLUIDS

**Principle.**—In this method (*Jour. Lab. and Clin. Med.* 23: 543, 1938) the alcohol is distilled in a closed container into standard dichromate. The excess unreduced dichromate is determined by titration from which the amount of alcohol can be calculated.

**Reagents.**—*Concentrated sulfuric acid.*

**Methyl orange solution.**—Place 0.1 gm. of methyl orange in a 100 cc. graduated cylinder. Add 25 cc. of  $\frac{N}{10}$  NaOH and shake to dissolve. Dilute with water to 100 cc.

**Ferrous sulfate solution.**—Dissolve 5.0 gm. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  in 15 cc. of water. Add 3 cc. of concentrated sulfuric acid and dilute to 25 cc.

**Reducing fluid.**—In a 50 cc. graduated mixing cylinder place 16 cc. of water and 16 cc. of concentrated sulfuric acid. Cool under running tap water. Add 15 cc. of the methyl orange solution, 1 cc. of the ferrous sulfate, dilute to 50 cc. and mix. This reducing fluid keeps but a few days; the solutions from which it is prepared keeps for several months.

**Standard dichromate solution.**—Dissolve 0.852 gm. of potassium dichromate and dilute to 1 liter in a volumetric flask (1 cc. of the solution is equivalent to 0.2 mg. of ethyl alcohol).

**Procedure.**—1. Pleat a  $2\frac{1}{2}$  inch square of filter paper into a compact form of about  $2\frac{1}{2} \times \frac{3}{8}$  inches and fasten by means of a paper clip to the center of the inside of a rubber stopper that fits a 100 cc. Erlenmeyer flask.

2. Place exactly 10 cc. of the standard dichromate solution in the flask, add 10 cc. of concentrated sulfuric acid and mix.

3. Pipet 0.5 cc. of blood or urine and while holding the stopper raised above the flask sufficiently to expose a portion of the filter paper, transfer the sample to the filter paper and place the stopper lightly in the neck of the flask.

4. Flame the neck of the flask briefly and stopper tightly.

5. Allow to stand overnight at room temperature, or 2 hours at 70° C. or 15 minutes in boiling water. Cool.

6. Pipet 10 cc. of the standard dichromate solution into another flask and add 10 cc. of concentrated sulfuric acid. Cool.

7. From a buret, add the reducing fluid, a small portion at a time, to each flask until a permanent pink color is obtained. Note the amount of titrating fluid used for each flask.

**Calculation.**—The amount of dichromate used is equivalent to 2 mg. of alcohol. If  $S$  = titration of standard and  $U$  = titration of unknown.

$$\frac{S - U}{S} \times 2 = \text{mg. of alcohol in sample and}$$

$$\frac{S - U}{S} \times \frac{400}{1000} \text{ or } \frac{S - U}{S} \times 0.4 = \text{per cent of alcohol in sample.}$$

**Notes.**—1. Use fresh samples when possible. However, if kept in the refrigerator no changes occur in alcohol content in 24 hours.

2. In bleeding the patient sterilization of the skin must be done with an aqueous solution of a germicide and not with alcohol.

### MOTLEY METHOD FOR THE RAPID DIFFERENTIATION OF THE BARBITURATES AND OTHER SEDATIVES

**Reagents.**—*Mercurous nitrate, 15 per cent.* Add 5 cc. of concentrated nitric acid (from a cylinder) to 95 cc. of water. Mix and add 15 gm. of mercurous nitrate. Add a few drops of metallic mercury. Shake until the salt is dissolved.

*Mercuric nitrate, 15 per cent.* Made up in 5 per cent nitric acid.

*Potassium iodide, 16.5 per cent.*

*Ferric chloride, 9 per cent.*

All the solutions should be kept in amber bottles and must be discarded when they become hazy or turbid.

**Procedure.**—1. Place 10 cc. portions of the unknown solution into 2 test tubes. To 1 tube add 1 drop of the mercurous nitrate solution and note the reaction; then add 2 drops of the potassium iodide solution.

2. To the other tube add 1 drop of mercuric nitrate solution and note the reaction; then add 2 drops of the ferric chloride solution followed by 2 drops of the potassium iodide solution.

3. Table 54 shows the results to be expected in the first and second tubes.

### DELMONICO METHOD FOR THE DETERMINATION OF BARBITURATES IN URINE

**Principle.**—In this method (*Proc. Staff Meet. Mayo Clinic*, 14: 109, 1940) the acidulated urine is extracted with chloroform and the color produced in the purified extract by cobalt acetate solution is compared with a solution of barbituric acid.

**Reagents.**—*Cobalt acetate, 1 per cent in absolute methyl alcohol*

*Isopropylamine, 5 per cent:* 5 cc. of isopropylamine (Eastman) diluted to 100 cc. with absolute methyl alcohol.

*Activated charcoal*

*Sulfuric acid, 10 per cent*

*Chloroform, U.S.P.*

*Standard Barbituric Acid:* Place 50 mg. of barbital in a 100 cc. volumetric flask. Dilute to volume with chloroform (1 cc. = 0.5 mg.).

**Procedure.**—1. Place 25 cc. of urine in a 125 cc. separatory funnel. Acidulate with 10 per cent sulfuric acid, add 25 cc. of chloroform and shake.

**TABLE 54.—DIFFERENTIATION OF BARBITURATES FROM OTHER DRUGS \***

Drug	Tube 2			
	Tube 1	Tube 2		
	HgNO <sub>3</sub>	KI	Hg(NO <sub>3</sub> ) <sub>2</sub>	KI
Barbital, phenobarbital, pentobarbital, amylal, alurate, evipal (sodium)	White-gray gel. ppt.	Green fine ppt.	White gel. ppt.	Clear
Ortal sodium	White gel. ppt.	Green fine ppt.	White gel. ppt.	Ppt. present
Seconal	White gel. ppt.	Green fine ppt.	White gel. ppt.	Hazy
Pentothal sodium	White-gray fine ppt.	Green-gray fine ppt.	White gel. ppt.	White fine ppt.
Dilantin sodium	White gel. ppt.	Green fine ppt.	White gel. ppt.	Ppt. present
Aminopyrine	Purple (fades)	Green coarse ppt.	Clear	Clear
Antipyrine	Gray fine ppt.	Slate gray ppt.	Clear	Wine red
Acetanilide				Yellow coarse ppt.
Acetophenetidin	Clear	Yellow fine ppt.	Clear	Clear
Sulfomethane	Clear	Gray fine ppt.	Clear	Clear
Sulfomethylmethane	Clear	Gray fine ppt.	Clear	Clear
Potassium and sodium bromide	White fine ppt.	Yellow fine ppt.	Clear	Clear
Sodium salicylate	Light gray gel. ppt.	Yellow coarse ppt.	White gel. ppt.	Clear
Aspirin, avertin, chloral hydrate	Clear	Yellow fine ppt.	Clear	Clear
Carbromal	Clear	Dark brown ppt.	Clear	Clear
Scopolamine HBr.	White fine ppt.	Yellow coarse ppt.	Clear	White fine ppt.
Heroin	White fine ppt.	Brown coarse ppt.	Clear	Gray ppt.
Morphine sulfate	Clear	Green coarse ppt.	Clear-green	Clear-green
Apomorphine HCl.	White ppt.-pink	Gray fine ppt.	Clear-red	Yellow turbid
Codeine phosphate	White fine ppt.	Green coarse ppt.	White fine ppt.	Yellow ppt.
Codeine sulfate	Clear	Green coarse ppt.	Clear	Yellow ppt.
Dilaudid HCl.	White fine ppt.	Green fine ppt.	Clear	White ppt.
Papaverine HCl.	White fine ppt.	Brown coarse ppt.	Clear	White ppt.
Strychnine, benzedrine				
Atropine (sulfate)	Clear	Green coarse ppt.	Clear	Clear
Cocaine HCl.	White fine ppt.	Green coarse ppt.	Clear	White coarse ppt.

\* Modley, H. L., *J. Missouri M.A.*, 38: 78, 1941.

2. Allow the chloroform layer to separate and draw it off into a 25 cc. glass-stoppered cylinder. Add about 0.5 gm. of activated charcoal and shake to remove pigment.

3. Decant the chloroform and concentrate it to 5 cc. Transfer to a test tube. Place 5 cc. of the standard in another test tube. Add to both tubes 0.25 cc. of the cobalt solution followed by 1.5 cc. of isopropylamine and mix.

4. Compare in the colorimeter at once. The sulfonamide compounds interfere with the color reaction.

### METHODS FOR THE DETECTION OF VERONAL, LUMINAL, MORPHINE, STRYCHNINE, BRUCINE, CODEINE AND COCAINE

**Principle.**—The above substances are separated by extraction, purified, and identified by one or more of the tests listed under each individual substance.

The procedure which follows is devised to identify one or more of the above when present in human organs, meat, vegetables, milk, potatoes and other substances.

**Reagents.**—*Erdmann's*: Add 10 drops of a mixture of 10 drops of concentrated nitric acid and 100 cc. of water, to 20 cc. of concentrated sulphuric acid.

*Froehde's*: Dissolve (by gentle heating) 5 mg. of molybdic acid or sodium molybdate in 1 cc. of concentrated sulphuric acid. This solution should be colorless. It does not keep well.

*Mandeline's*: Dissolve 1 part of finely ground ammonium vanadate in 200 parts of concentrated sulphuric acid.

*Marquis*: Mix 2 to 3 drops of 40 per cent formaldehyde solution with 3 cc. of concentrated sulphuric acid. Prepare this solution when needed.

**Preparation of Material for Extraction.**—1. Mix a quantity of finely ground material in a large flask and mix with 2 to 3 times as much absolute alcohol. Add enough tartaric acid (10 per cent) to make the mixture acid to litmus. Avoid a large excess of tartaric acid.

Using a Liebig condenser as a reflux, heat the flask on a water-bath for 15 to 20 minutes at 60° C.

2. Cool the flask and contents and filter off as much fat as possible. Rinse the residue with alcohol, filter and combine the filtrates. Should the filtrate fail to be acid, acidify with tartaric acid.

3. Evaporate the filtrate to a thin syrup on a water-bath and mix the residue with 100 cc. of cold water.

4. Filter through moistened filter paper and evaporate to a syrup on a water-bath.

5. Mix the residue thoroughly with liberal quantities of absolute alcohol. Filter. Evaporate the filtrate on a water-bath and dissolve the residue in about 50 cc. of water. Should this solution fail to be perfectly clear, filter through moistened filter paper. This filtrate (or original solution) should have an acid reaction. For convenience in future reference to this solution, it is designated as "Solution A."

**Extraction and Identification of Veronal and Luminal**—1. Extract "Solution A" several times with ether. Evaporate the ether extract to dryness. (Save acid aqueous solution for possible tests described below.)

2. Recrystallize the ether extract residue from a small quantity of hot water, using animal charcoal to remove color.

3. The identification of veronal and luminal can be made on the basis of the following properties:

(a) *Color Reaction*.—This test identifies the barbiturates as a group, and is not specific for veronal or luminal. To each of 3 tubes (No. 1, No. 2, No. 3), add some chloroform solution of the suspected purified crystals. To each add 1 to 2 drops cobalt acetate (1 per cent) in methyl alcohol. To tube No. 1 add 2 drops of barium hydroxide in methyl alcohol; to tube No. 2 add 4 drops and to tube No. 3 add 8 drops. A blue color in any of these tubes constitutes a positive test for the barbituric group.

(b) *Melting-Point*.—This determination is important because it serves as practically the only reliable means of differentiating between individual barbiturates.

The melting-point of pure veronal is  $188^{\circ}$  to  $191^{\circ}$  C. When impure it melts at  $187^{\circ}$  to  $188^{\circ}$  C.

The melting-point of luminal is  $171^{\circ}$  to  $173^{\circ}$  C.

(c) *Sublimation*.—Veronal when heated cautiously, easily sublimes.

**Extraction and Identification of Morphine.**—1. "Solution A" is made strongly alkaline with sodium hydroxide solution and extracted with ether. Most alkaloids, except morphine, are in the ether extract. Save the ether extract for possible tests under IV.

2. Make the alkaline aqueous solution acid with dilute hydrochloric acid and then make alkaline with sodium bicarbonate.

3. Morphine is extracted with a hot solution consisting of 9 volumes chloroform and 1 volume absolute alcohol. (Method of Kippenberger.)

4. Pour the combined chloroform extracts into a dry vessel, add a few crystals of anhydrous sodium sulphate to remove any water which may be present and filter the chloroform extract (dry filter) into a small vessel. Evaporate to dryness.

5. Purification: The residue is dissolved in hot amyl alcohol and shaken thoroughly with water acidified with dilute sulphuric acid. The aqueous extract is then made alkaline with ammonia, and extracted several times with hot chloroform. Evaporate to dryness.

(a) Morphine appears as white crystals, the melting-point of which is  $230^{\circ}$  C.

(b) Morphine is strongly alkaline and can be titrated.

(c) With Mandeline's reagent the color changes are red violet, to blue, to violet.

(d) With Froehde reagent the color changes are violet, to blue, to green.

(e) *Physiological test*: A neutral solution of morphine (HCl) when injected under the dorsal skin of a white mouse causes the tail of the animal to become S shaped. Five hundredths of a milligram of morphine will maintain the tail in this position for 45 minutes. Five milligrams will maintain it for 20 hours.

**Extraction and Identification of Strychnine, Brucine, and Codeine.**—"Solution A" is made strongly alkaline with sodium hydroxide solution and extracted 3 to 4 times with ether. Set the combined extracts aside in a dry flask (loosely stoppered) for 2 to 3 hours. Decant through a dry filter and evaporate from a small dish on a water-bath which has been previously warmed. The residue will contain *Strychnine*, *Brucine*, and *Codeine*.

*Identification of Strychnine.*—1. Crystals melt at  $268^{\circ}$  C.

2. With Mandeline's reagent the color changes are blue to blue violet to red violet to red, and in 15 to 20 min. to orange. If the orange color fails to appear Strychnine is *not* present.



3. Erdmann's and Froehde's reagents give no color change with strychnine.

*Identification of Brucine.*—1. Color changes using Erdmann's and Froehde's reagents may be employed to identify Brucine.

2. Erdmann's reagent gives a red color which changes to yellow.

3. Froehde's reagent also gives a red color which changes to yellow.

*Identification of Strychnine in the Presence of Brucine.*—1. Brucine when present together with strychnine may render inconclusive the tests for the latter. To avoid this the brucine can be destroyed before applying the strychnine tests.

2. The residue (or part of) containing brucine is dissolved in about 2 cc. of dilute sulphuric acid, 2 drops of concentrated nitric acid are added, and the system permitted to stand for 2 to 3 hours. It is then made strongly alkaline with sodium hydroxide solution and extracted with ether. The evaporated ether extract will contain strychnine, but not brucine which is destroyed by this procedure.

*Method for the Identification of Codeine.*—1. When codeine is warmed with Erdmann's reagent a blue color results.

2. With Froehde's reagent there is obtained a yellow green color which changes to blue.

3. Mandeline's reagent with codeine gives a green color which changes to blue.

*Method for Differentiation between Morphine and Codeine.*—1. On the addition of an aqueous solution of ferric chloride: A blue color is obtained with *morphine*. No change takes place in the case of *codeine*.

2. On the addition of hydrogen iodide: Iodide is rapidly liberated in the case of *morphine*. No iodine is liberated in the case of *codeine*.

*Method for the Extraction and Identification of Cocaine.*—"Solution A" is made alkaline with sodium bicarbonate solution and extracted with ether. Evaporate to dryness and apply the following tests for the identification of cocaine:

1. When cocaine is warmed with Marquis reagent, a wine red color is obtained.

2. Add a small amount of sulphuric acid (conc.) and a crystal of potassium iodate to the suspected cocaine. At this point there is no visible change, but when the solution is heated until  $\text{SO}_3$  fumes appear the following color changes take place: Brown, to olive, to blue, to violet, which finally fades.

3. Add a small amount of sulphuric acid (conc.) and place on a water-bath for 5 minutes. Then carefully add water. If cocaine is present there is obtained the odor of methyl benzoate (Oil of Peppermint).

## METHOD FOR THE DETECTION OF BROMINE IN URINE

**Principle.**—Urine is ashed with alkali. From the resultant bromides, bromine is liberated and finally concentrated in chloroform, with the production of a brown color.

**Procedure.**—1. In a nickel crucible place about 50 cc. of the suspected urine. Add 1 gm. of sodium carbonate and 0.5 to 1 gm. of potassium nitrate and evaporate to dryness. (The capacity of the crucible employed should be approximately twice the volume of the sample taken for analysis.)

Remove adherent particles of carbon by sprinkling powdered potassium nitrate over the dried residue. During this latter operation rotate the crucible and continue heating.

2. Permit the system to cool to room temperature, add 10 to 15 cc. water to the

ash, and make distinctly acid (to litmus) with dilute sulphuric acid. The solution at this point is, as a rule, turbid.

3. Transfer to a separatory funnel, rinsing the crucible with 10 to 20 cc. of acid potassium sulphate (10 per cent).

4. Add 20 to 30 cc. of chloroform, followed by a drop by drop addition of potassium permanganate (3 per cent) until the color no longer disappears. Finally an excess of 15 drops of permanganate is added.

5. Stopper the cylinder and place in the dark for 15 min. Shake well and permit the brown solution of bromine in chloroform to settle.

### DIETHELM METHOD FOR THE DETERMINATION OF BROMIDE IN BLOOD

**Principle.**—In this method (*Jour. Nerv. and Mental Dis.* 71: 151, 1930) the color produced by the reaction of bromine with gold chloride is measured colorimetrically.

**Reagents.**—Gold chloride, 0.5 per cent

Trichloroacetic acid, 5.0 per cent

**Sodium chloride-trichloroacetic acid solution:** To 0.12 gm. of sodium chloride in a 100 cc. cylinder add 70 cc. of the trichloroacetic acid. Dilute to 100 cc.

**Stock standard:** An accurate 1 per cent solution of sodium bromide (1 cc. = 10 mg.).

**Dilute standard:** Pipet 5 cc. of the stock standard into a 100 cc. volumetric flask. Dilute to the mark with the sodium chloride-trichloroacetic acid mixture (1 cc.: 0.5 mg.).

**Procedure.**—1. In an Erlenmeyer flask place 16 cc. of trichloroacetic acid solution. Add slowly, with shaking, 4 cc. of serum. Mix and allow to stand 30 minutes. Filter.

2. Place 10 cc. of clear filtrate in a test tube.

3. Place 4 cc. of dilute standard in another test tube and add 6 cc. of sodium chloride-trichloroacetic acid mixture.

4. Place 8 cc. of dilute standard in a third tube and add 2 cc. of the same diluting fluid.

5. Place 10 cc. of the sodium chloride-trichloroacetic acid in a fourth tube.

6. Add 1.0 cc. of gold chloride solution to each tube and mix.

7. Compare in the colorimeter with the nearest standard.

**Calculations.**—Using the equivalent of 2 cc. of blood serum, the first standard is equivalent to 100 mg. and the second standard to 200 mg. of sodium bromide per 100 cc. Set the standard at 10; then mg. per cent of sodium bromide is

$$\frac{1000}{\text{reading of unknown}} \text{ or } \frac{2000}{\text{reading of unknown}}$$

**Notes.**—1. If a photoelectric colorimeter is available, use light filter No. 54 and the fourth tube as a blank; subtract the reading from the readings of the unknown and standards.

2. Bromide concentrations in excess of 150 mg. per cent are found in bromide intoxications.

3. If the bromide concentration exceeds 250 mg. per cent the test should be repeated with smaller amounts of sample.
4. Iodides form a brown precipitate with gold chloride.

### GREENBERG METHOD FOR THE DETERMINATION OF BROMIDE IN BLOOD

**Principle.**—In this method (*Jour. Lab. and Clin. Med.* 28: 779, 1943) the bromide of the deproteinized whole blood is oxidized to bromate and determined by iodometric titration.

**Reagents.**—*Zinc sulfate, 0.45 per cent*

*Sodium hydroxide, 0.1N*

*Hydrochloric acid (2N):* Dilute 197 cc. of concentrated hydrochloric acid to 1 liter.

*Calcium hypochlorite:* A 15 per cent solution of  $\text{Ca}(\text{OCl})_2$  is filtered and the filtrate diluted with 3 parts of water.

*Calcium carbonate powder*

*Sodium formate, 20 per cent*

*Potassium iodide, 10 per cent*

*Starch solution*

*Sodium thiosulfate (0.0004N):* Dilute 4.0 cc. of 0.1N sodium thiosulfate to 1 liter.

**Procedure.**—1. Place 6.5 cc. of the zinc sulfate solution in a test tube.

2. With a special 0.1 cc. Folin micro-blood pipet collect 0.1 cc. of blood from an ear lobe or finger tip and discharge it directly into the solution, rinsing the pipet 2 or 3 times with the same solution.

3. Add 0.5 cc. of the 0.1N sodium hydroxide, shake the tube, place in boiling water 3 minutes and cool.

4. Filter and place 5 cc. of filtrate into another test tube.

5. Add in succession, 0.3 cc. of the 2N hydrochloric acid, 1.0 cc. of the hypochlorite solution and a pinch of the calcium carbonate powder. The carbonate should be in excess as indicated by its persistence as a precipitate.

6. Place the tube in boiling water for 8 minutes, add 0.4 cc. of the 20 per cent sodium formate and heat 8 minutes longer. Remove from the water bath and allow to cool.

7. Add in succession 3 cc. of the 2N hydrochloric acid and 1.0 cc. of the 10 per cent potassium iodide solution; shake.

8. Add 0.5 cc. of the starch solution and then the standard thiosulfate solution, drop by drop, from a buret until the blue color is just discharged.

9. Make a blank determination whenever new reagents are prepared. The blank ordinarily ranges from 1.0 to 1.7 cc. of thiosulfate.

**Calculation.**—Each cubic centimeter of 0.0004N thiosulfate corresponds to 7.6 mg. of bromide or 9.5 mg. of sodium bromide per 100 cc. of blood. Therefore, subtract the blank titration from the titration of the unknown and multiply the difference by either 7.6 or 9.5.

**TEISCHMANN'S TEST FOR BLOOD STAINS**

**Principle.**—The test depends on the fact that hematin is formed from the decomposition of the hemoglobin by heat, and secondly, that the hematin in solution in boiling glacial acetic acid unites with the chlorine of the salt to form chloride of hematin, which is soluble in boiling glacial acetic acid, crystallizing from this solvent on cooling.

**Procedure.**—If the material is a dried blood stain, a small fragment of the dried blood should be removed from the stain with the point of a knife and transferred to a glass slide. If the stain be a diffused one, or if the blood, while still fresh, has soaked into the fabric, as in the case of a stain on cotton or linen cloth, then it suffices to scrape a small portion of the stain with the knife point, collecting the dust thus removed on a glass slide. The fragment of dried blood or the dust should then be treated on the slide with a small drop of water in which has been dissolved a minute fragment of sodium chloride. This drop should then be evaporated to dryness by gentle heat, the dried residue covered with a cover glass, a drop of glacial acetic acid allowed to run under the cover glass, and the slide again gently heated until bubbles of gas are seen to form in the liquid under the cover glass. This shows that the glacial acetic acid has been heated to the boiling point. If, now, the slide be allowed to cool, the microscope will reveal the characteristic crystals of chloride of hematin in case the stain examined contained blood. These crystals of chloride of hematin are called "hemin" crystals, and they have a characteristic form (Fig. 336).



FIG. 336.—HEMIN CRYSTALS (WOOD)

The normal hemin crystals have a yellow to chocolate-brown color and separate in the form of small rhombic plates. They naturally vary a little in size according to the rapidity of their formation. Sometimes, particularly if the fragment of dried blood on the slide was of considerable size, the form of the crystals in some parts of the prepa-

ration may be somewhat modified, some assuming a pointed, oval shape, and in some the outlines may be a little irregular; in all cases, however, a sufficient number of the normal perfect crystals will be seen to render their identification positive.

**Precautions.**—1. Care should be taken in heating the slide not to raise the temperature so high as to decompose the hematin in the first dry residue obtained. If the temperature be raised to about  $142^{\circ}\text{C}$ . ( $287.6^{\circ}\text{F}$ .), no hemin crystals will be formed.

2. On further heating the slide, after the addition of glacial acetic acid, the temperature should not be raised so high as to produce active boiling of the acid, since active ebullition may carry all the pigment beyond the edge of the cover glass, which might prevent the detection of the hemin crystals.

3. The hemin test will not detect blood pigment in blood stains that have been heated to a high temperature, that have been subjected to the prolonged action of naphtha, solution of ammonium chloride, or bromochloralum, or that have been exposed for a long time to direct sunlight.

4. This is by far the most important test for blood pigment, and it is extremely delicate. While the detection of hemoglobin shows with certainty the presence of blood, it throws no light upon the nature of the animal from which the blood came. To determine this latter question resort is had to microscopic examination of the blood cells and especially to the complement fixation and precipitin tests.

# PATHOLOGICAL METHODS

## METHODS FOR THE MICROSCOPICAL EXAMINATION OF TISSUES

FRANK W. KONZELMANN

Histologic or tissue technic is an art that is mastered only by patience and experience. It is the one field in pathology wherein these two qualities and the willingness to strive for perfection are of paramount importance. Accuracy in pathologic diagnosis requires perfectly prepared tissue sections as well as a broad experience and a knowledge of the principles of pathology. It cannot be hoped for with either alone, yet one observes that the most experienced pathologist is the most exacting in matters of technic while the least experienced is often satisfied with preparations of the poorest quality.

### EQUIPMENT

Apparatus of the best grade, properly cared for and reagents carefully prepared are prerequisites of good technic.

The chief apparatus of the histologic technician consists of the microtome and its accessories, the paraffin oven, staining dishes and jars, and the microscope.

**The Microtome.**—There are many types. The Spencer rotary microtome is the best for paraffin embedded tissue. The microtome should never be taken apart except by a mechanic trained in this field. It should, however, be frequently cleaned by wiping all the accessible moving parts with a cloth moistened with xylol and well lubricated by a good grade of paraffin oil furnished by the manufacturer. Each day after it has been used, it should be cleaned of paraffin (a good stiff-haired one inch painter's brush is handy for this purpose). All water must be removed from the crevices of the knife holder and chassis. After it is thoroughly dried, a little "Three in One" oil may be rubbed over the exposed parts to prevent rusting. The instrument should be covered with a bag or bell jar.

The sliding microtome may be used for paraffin sections, especially for those greater than 2 cms. in diameter. The knife must be set so that its long axis forms a true right angle with the direction of its cutting motion. It must be parallel, in other words, with the trimmed edge of the paraffin block. Those types in which the knife is supported at both ends are the best. The knife is held by a carriage which rides upon a well-lubricated track, while the tissue block remains stationary. This is the best type of microtome for celloidin embedded tissues. When the latter material is cut, the knife is set at an oblique angle so that as much of its cutting edge as possible is utilized. Its long axis, in other words, forms an angle of 45 degrees or less with the direction of its cutting motion.

**The Freezing Microtome.**—Whenever for some reason tissues are not embedded in either paraffin or celloidin, they may be cut in sufficiently thin sections with the freezing microtome (Fig. 337). The tissue, never more than 1 cm. in diameter, is placed upon the freezing chamber and frozen by means of ether spray or liquid carbon dioxide. The latter is the quickest and most satisfactory agent. It is available in large tanks which can be conveniently attached to this special type of microtome. Freezing chambers may be attached to the sliding microtome and used successfully. The Spencer Freezing microtome is an excellent instrument though the author prefers the older type made Sartorius microtome, the knife of which is supported at both ends in a far more stable manner. The same care must be given these microtomes as prescribed for the rotary type. It is to be emphasized that good sections cannot be obtained with a worn out microtome.

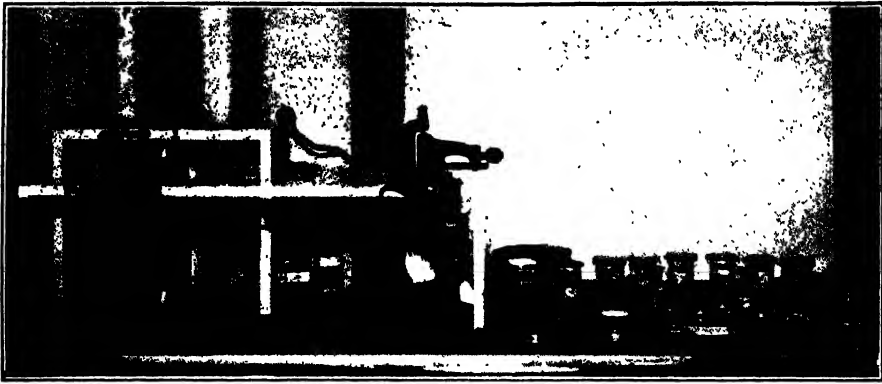


FIG. 337.—THE FREEZING MICROTOME ARRANGEMENT SHOWING THE H-TUBE BY WHICH EACH MICROTOME MAY RECEIVE  $\text{CO}_2$  FROM EITHER CYLINDER

**Microtome Knives.**—These are the Waterloo of many a technician. All other requirements for good technic may be met, yet a poor knife will bring about complete failure. Good steel is the first essential. Knives sold by the leading optical companies seem to be of the finest steel. Those made by Spencer in the past have been too soft. They are easily sharpened but do not retain their edge. Knives imported from Germany in the years before the World War were too hard. It was extremely difficult to obtain a good cutting edge and nicks occurred more frequently than with the softer steel. Spencer's recently made knives and those of Bausch and Lomb or Arthur H. Thomas in Philadelphia seem to be of a desirable degree of hardness.

The practice of sending knives out to a grinder to be sharpened is not recommended unless it be for the removal of deep nicks or to "true" a knife that has been ground irregularly. The knife should be frequently honed and stropped if its edge is to be maintained. The microtome knife sharpener of Schmidt or Fanz may be used successfully. One or the other should be in every laboratory and its care delegated to one individual. The directions for the use of either are fully given by the dealer who supplies them. The author has for years used the smaller hones and while good results are obtained, expertness is necessary. A honing guide must be fitted to the back of each knife and so marked that it is always placed upon the knife in the same manner. A medium coarse yellow Belgian hone is used for rapid grinding out of small nicks.

The stone must be constantly flooded with water lest the particles of steel removed roll under the edge and produce additional nicks. Final honing is performed on a fine blue-green stone. The surface of this stone must likewise be flooded with water. A small accessory stone is provided with each hone for the purpose of keeping the surface flat and free of scratches. It should be rubbed upon the hone evenly (20 or 30 strokes) each time before the hone is used. The knife should be placed flat upon the distal end of the hone and drawn towards the worker cutting edge foremost, without pressure and bringing the whole cutting edge of the knife across the hone. As the mechanic expresses it, the knife is drawn from heel to toe (point). The knife is turned on its back (never the edge) and the opposite side is honed in the same way. These 2 motions are repeated until the desired edge is obtained (Fig. 338).

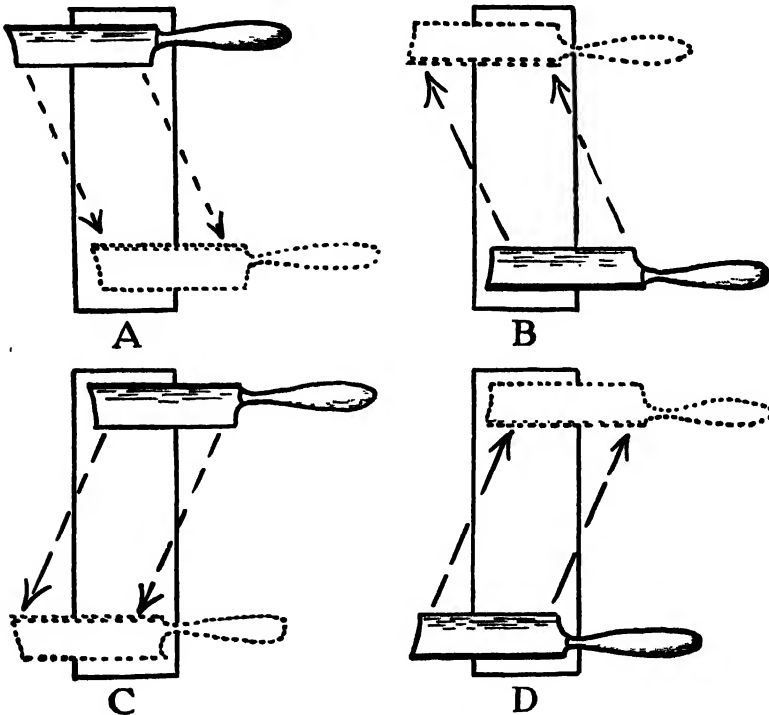


FIG. 338.—METHOD FOR HONING MICROTOME KNIVES

The first motion is as above described from heel to toe then, turning the knife on its opposite side, it is ground from toe to heel. It is turned again and ground this time toe to heel and the fourth motion after turning it from heel to toe. Thus each side of the edge is ground with a criss-cross motion. This serves to prevent a belly in the cutting edge and seems to increase the keenness of the edge. It is well to study the effect of the honing by frequent examination of the edge under the microscope using the 16 mm. objective. A good edge must be quite even and free of nicks. The scratches formed by the rough hone must be polished out by the fine hone and the strop.

**Stropping.**—Grinding or honing results in the formation of serrations like the teeth of a saw, in the cutting edge. These are extremely fine when the knife is properly



honed. The purpose of the strop is to bring these serrations into a straight line. The flexible barber's strop of the best grade is most desirable although the flat mounted strops seem more popular. Strop dressing is not needed unless the principle of the barber be followed in occasionally rubbing in a quantity of shaving soap. This serves to keep the leather soft and pliable. Gouges and nicks in a strop are an evidence of inexcusable carelessness and indifference. Occasional stropping will keep a good knife in excellent condition for the cutting of many paraffin blocks.

A razor is a necessary instrument for trimming pieces of tissue. The old-fashioned straight type is the best. Its edge should be constantly maintained. The practice of using razor blades may be convenient but they are a needless expense. Many times a longer sturdier blade is needed.

**The Paraffin Oven.**—Any type which will maintain a constant temperature will prove satisfactory. The electrically heated ovens are the safest, for open flames are a source of danger whenever such fluids as xylol, benzol or acetone are being employed. Dioxane, a dehydrating agent recently recommended, especially demands an electrically heated oven. Those fitted with a paraffin well have never proved satisfactory for the spigots have always leaked. The paraffin used for blocking may be kept in a beaker or enamel pitcher on a shallow tray in the oven.

**Glassware.**—The Coplin type of staining jar is the most satisfactory. Large Petri dishes of about 6 inches diameter and three inch depth may be used when filled with warm water to receive paraffin section after cutting. The smaller varieties are suitable for staining celloidin sections. In place of the former a large pyrex baking dish, its bottom painted black, mounted in a suitably sized box, serves to receive paraffin sections. The ordinary 75 watt electric bulb mounted in the box will warm the water in the dish sufficiently to flatten out paraffin sections. Small slender dishes are convenient for staining frozen sections. Their glass covers prevent the evaporation of stains.

## RAPID METHODS FOR PREPARING SECTIONS OF TISSUE

**Ultropak Method.**—The most rapid method of preparing tissue for microscopic study is that using the Ultropak microscope. This instrument utilizes an objective built in an illuminator so that the light is reflected from above down upon the tissue on the microscope stage. The tissue, which should be fresh, is prepared by cutting a thick slice (1 to 3 mm.) with a sharp straight razor so that a plane even surface is obtained. The freshly cut surface is dipped in a thin layer of 0.3 per cent solution of toluidine blue (Grübler) for 20 to 30 seconds and quickly washed with acidulated water. It is immediately placed upon the stage and examined. The tissue must be kept moist. The polychrome effect renders sharp differentiation between cells and stroma. After some experience one can diagnose malignancy as accurately as by the more tedious frozen method. Further, it permits the examination of larger areas.

**Terry's Method.**—The procedure just described has the disadvantage of requiring an expensive objective. Terry has devised a method wherein the ordinary microscope may be used. A thinner slice (0.5 to 1 mm. thick) is cut, with as before, a plane even surface. It is placed upon a microscope slide and Grübler's or Terry's modified polychrome methylene blue is painted on the exposed surface of the tissue. Staining must be done quickly (about 30 seconds) and care must be taken that no stain runs between the tissue and the glass slide. The tissue is flooded with water, a coverglass is placed

over it and it is now ready for study. Here, only the superficial layer of cells is stained. The section is illuminated by the condenser of the microscope in the usual way. It is thin enough so that light passes through the unstained tissue readily. Considerable nuclear detail may be seen in these sections. The method is as adaptable as the previous one and reveals finer detail. With Terry's original method it is quite difficult to cut a thin slice possessing a plane surface. Unfixed tissue is apt to be particularly soft and difficult to cut. If unevenness of surface occurs a clear image cannot be obtained. Konzelmann uses a hemostat or a specially designed handle in which 2 razor blades are supported. The long Durham Duplex blade serves best. With the hemostat, the blades are separated by a narrow strip of cardboard of a thickness equal to that desired of the section (0.5 to 1 mm.). The cardboard should be as long as the blade and about 3/16 of an inch wide. It is placed along one edge between the 2 blades and then the whole grasped and locked firmly in the jaws of the hemostat. Thus a double-edged knife is formed. It is drawn downward into the tissue with an even sweeping motion. When it has entered the tissue to its full depth, the blades are tilted almost at a right angle to their former position and by a slight sawing motion the thin slice between the blades is severed from its point of attachment. The blades may then be separated and the section transferred to the slide. This procedure may be carried out routinely while another technician is preparing the frozen section. Often a diagnosis can be made before the tissue is completely frozen.

More recently, Terry has suggested placing the thin slice of tissue upon a thin slab of fine sponge (1 x 2 x 1/2 inches) wet with water. The stain is sprayed on the tissue from an inexpensive perfume atomizer. After a minute or more, depending on the intensity of the staining reaction, the dye may be flushed off with water. A specially prepared glass microscopic slide is used. It is bound by adhesive tape or water-proof glue to a thin strip of wood of the same size. An oval or circular hole 3/4 inch in diameter is cut in the wooden strip. This slide is touched to the section of tissue flatly with the wooden strip down. The section will adhere to the glass and may then be examined microscopically through the slide.

**Frozen Sections.**—If the sections are to be cut quite thin (1/100 of a millimeter—10 microns, or less) the tissue must be rendered firm. In the slower methods this is brought about by infiltration with paraffin or celloidin. The tissue is altered by the solutions through which it is passed so that it neither looks the same microscopically nor is it fit for certain special stains. Fat, of course, would be dissolved by the dehydrating fluids or clearing agents. Hardening the tissue by freezing is a method to be chosen where immediate sections are desired or whenever the solutions used in the slower methods render the tissue unfit for certain special studies.

Various freezing agents have been used but liquid carbon dioxide is probably the most efficient and convenient. Recently, carbon dioxide snow has been suggested by Lindsay and his colleagues and probably is just as serviceable. For the latter method a special block had been designed (Fig. 339) which may be used in a simple microtome to hold the tissue in contact with the "dry ice." The method is well worth considering because of its convenience. The liquid carbon dioxide method is the method of choice. The freezing agent is supplied in iron tanks or cylinders; several should be kept on hand for there is no way of telling when the one in use is nearly empty. It ceases to function suddenly. It is a wise custom to have 2 separate microtomes connected to separate cylinders or to connect 2 tanks to a single microtome. If one becomes ex-

hausted, its valve may be closed and the second tank opened with negligible interruption. The cylinder should be mounted valve end down at an angle of 45 degrees or better still, on its end. It is the liquid carbon dioxide, in its transformation to a gas, that brings about the freezing. If only the gas is released as would occur when the cylinder is mounted valve up, there would not be sufficient heat extraction to freeze the tissue.

The tissue may be either fresh, unfixed, or fixed in hot (60° C.) formalin for 3 to 4 minutes. Fresh tissue is frequently difficult to cut and to handle after it is cut but some stains will be effective only with fresh tissue. Cold formalin requires several hours to penetrate so if immediate sections are demanded, hot formalin must be used. It does cause shrinkage but the alteration is not great enough to defeat the purposes of this rapid method. The block of tissue should not be more than 3 mm. thick, nor more than 1 cm. on a side.



FIG. 339.—THE "DRY-ICE"  
CHAMBER OF LINDSAY *et al.*

A little water is placed upon the freezing chamber, enough to form a pool about 2 mm. deep; the valve of the microtome is opened for a few seconds. The liquid thus released is immediately vaporized in the freezing chamber from which it issues in white clouds. Alternate releasing of the valve with intermissions of 5 or 10 seconds, conserves the freezing agent and is more effective than prolonged release. After the pool is *almost* completely frozen, the thin layer of unfrozen surface water should be wiped off with the finger. A flat instead

of rounded surface is obtained to receive the tissue. This thin layer of ice protects the knife lest it strike the metal platform after the tissue has been cut entirely through. Orient the tissue squarely so that the first contact with the knife will be with one of the corners of the block. If the knife strikes parallel to one of the sides of the block, the tissue will in all likelihood be dislodged. The tissue must be placed down in its proper position for as soon as it strikes the ice it will adhere and cannot be moved to a new position. After orientation cover the tissue with a little water and release the valve spasmodically until the tissue is frozen solid. If its surface is not now quite plane, it may be trimmed with a sharp knife or razor kept for that purpose. This conserves the edge of the microtome knife. Adjust the automatic feed to 10 microns.

Be certain that the surface of the tissue is 1 or 2 mm. below the edge of the knife and then with the right hand moving the knife in its sweeping stroke and the left advancing the micrometer screw  $\frac{1}{4}$  of a turn before each stroke, the tissue gradually comes into the plane of the knife edge. As soon as it begins to cut, permit the automatic feed alone to raise the block. Continue until the knife cuts the entire surface. The sections until now will in all likelihood be shredded or powdery because the tissue is too cold. It may be made warm by applying the finger for an instant and then cutting again. When the correct degree of hardness is brought about by the gradual warming of the tissue, the sections will cut easily and curl up slightly upon the knife, from which they may be lifted with a camel's hair brush. If the knife is brought over the tissue with a swift even stroke that terminates suddenly but not too firmly as the knife carriage strikes the post which limits the movement, the section will be thrown

from the knife. It may be caught in a dish of water which is held by the left hand to receive it. By either method the section is received in water or in 1 per cent salt solution and may then be carried through any of the staining technics. A number of sections may be cut in a short time and there will be plenty of tissue available for staining. Usually it is not necessary to freeze again but if the tissue becomes too soft it may be done as at first. Sometimes when the tissue is lacy and breaks easily it is better to permit it to become softer and to allow the sections to pile up on the knife edge. After 8 or 10 have thus accumulated the whole mass can be wiped off with the finger and transferred to the dish of water. They will then slowly separate and can be unfolded by gentle poking with a small brush. The separate sections may now be transferred to a staining dish with a section lifter or a camel's hair brush.

If the tissue is very fragile it is well to wash out the fixative and infiltrate before freezing with some mucilaginous medium made as follows:

Saturated aqueous solution of cane sugar.....	3 parts
Gum acacia mucilage .....	5 parts

Gum acacia mucilage is made by dissolving 60 grams of gum acacia in 80 cc. of distilled water.

The tissue is placed upon the platform in a pool of this mucilage as described for water above and frozen. The gum solution should be removed from the sections by washing in water before staining.

For rapid staining the following method may be employed:

Transfer the sections to a small dish containing Unna's polychrome methylene blue (Grübler) or Terry's modified polychrome methylene blue. The author has obtained good results with old solutions of Löffler's methylene blue. Keep the sections moving constantly for 10 to 20 seconds and then wash in 1 per cent sodium chloride solution.

Transfer to a slide and mount in glycerin jelly or Brunn's glucose medium, made as follows:

Glucose .....	240 grams
Distilled water .....	840 cc.
Spirit of camphor.....	60 cc.
Glycerin .....	60 cc.
Filter	

Dissolve the glucose in the water with gentle heating.

These sections are not permanent. Permanent sections may be prepared almost as rapidly. The section may be stained in dishes as above, using a small stender dish or watch glass for each solution or the section may be stained upon the slide. After transferring to a slide, drain the excess water and dehydrate with 80 per cent and then absolute alcohol. Permit a few drops of a mixture of equal parts of ether and absolute alcohol to flow over the section and then cover with 1 drop of very thin celloidin (0.05 per cent). Thin celloidin for this purpose may be prepared as follows:

1. Stock solution: One gram of celloidin in 50 cc. alcohol and ether mixture.
2. Add 5 cc. of this stock solution to 50 cc. of alcohol and ether mixture.

A thin film fastens the tissue to the slide but does not interfere with staining. The section may now be stained in hematoxylin and eosin as are paraffin sections. They

may be studied while still in creosote if protected by a cover glass or they may be at once permanently mounted in balsam.

### METHODS FOR THE FIXATION OF TISSUES

Fixation is the process whereby tissues are prevented from undergoing any disintegrative or digestive change. There is no ideal fixing agent. Many cause shrinkage of the tissue.

**Zenker's Fluid.**—Potassium bichromate ..... 2.5 grams  
 Mercuric chloride ..... 8 grams  
 Water ..... enough to make 100 cc.  
 Add 5 per cent glacial acetic acid before using.

The mercuric chloride will not all dissolve. The amount indicated will maintain a saturated solution which is required. Thin slices of tissue (not over 5 mm.) are fixed in 10 times their volume of the reagent for 12 to 24 hours. It is well to place a thin layer of cotton or paper on the bottom of the vessel so that the tissue does not stick to the glass. After fixation, wash the tissue for 24 hours in running water.

This reagent preserves well the structure of the tissue. Fibrin, fibrils and nuclear details are sharply defined when this fixative is followed by phosphotungstic acid hematoxylin. Tissues fixed in Zenker's fluid require longer hematoxylin staining time than those fixed in other reagents.

**Helly's Fluid.**—This is similar to Zenker's; 5 per cent formalin is added instead of glacial acetic acid. It preserves some types of cytoplasmic granules which are dissolved by acetic acid. It has the same advantages as Zenker's fluid and it is used in the same manner.

**Flemming's Solution.**—Osmic acid (2 per cent aqueous solution) . . . 4 cc.  
 Chromic acid (1 per cent aqueous solution) . . 15 cc.  
 Glacial acetic acid ..... 1 cc.

It is best to make this solution fresh from the aqueous solutions just as it is needed. It penetrates slowly and therefore tissues should be not more than 2 mm. thick. Complete fixation requires 1 to 2 days. The tissue must be washed for 12 to 24 hours before dehydration. This is an excellent fluid for the preservation of fat in tissue. The fat is blackened by the osmic acid.

**Alcohol.**—This reagent in 95 per cent or better, 100 per cent concentration is a good fixative but it causes considerable shrinkage unless it is used in ascending concentrations starting with 70 per cent. It hardens the tissue considerably. It is seldom used except where glycogen is to be demonstrated. Glycogen is very soluble in water and requires that the tissue be fixed in absolute alcohol and embedded in celloidin.

**Formaldehyde.**—Formaldehyde is a gas, soluble in water. It is obtainable in 40 per cent solution commonly known as formalin or formol. It is used in 4 per cent strength of the gas (10 per cent of the commercial solution). Formic acid is generated in formalin solutions and has a deleterious effect upon tissue. Therefore, stock bottles of the prepared fixative should contain a thick layer of marble chips on the bottom for the purpose of neutralizing it. Formalin penetrates rapidly and fixes well. It hardens the tissue. It permits the use of a wide variety of stains. It is the best fixative

for nerve tissue. It does not prevent shrinkage of tissue by alcohol in dehydration or by the heat of paraffin oven as well as Helly's or Zenker's fluids.

**Formol-Alcohol.**—A mixture of these 2 reagents has the advantage of fixing and dehydrating at the same time. There is considerable shrinkage of tissue which, however, does not greatly interfere with routine studies. It is frequently used for rapid paraffin or celloidin methods. The formula used at the Temple University Hospital is as follows:

Formalin (40 per cent formaldehyde solution) . . . .	18 cc.
Alcohol (95 per cent) . . . . .	60 cc.
Glacial acetic acid . . . . .	3 cc.
Water . . . . .	39 cc.

Pieces of tissue from 2 to 4 mm. thick are fixed in 1 to 4 hours.

**Picro-Formol-Acetic Acid Mixture.**—These mixtures are well known because of the excellent manner in which they preserve cytologic details. Some nuclear aniline dyes do not stain well after fixation in this fluid. If Bouin's fluid or a modification is used some tissues should be fixed in Helly's or Flemming's fluid.

<b>Bouin's Fluid.</b> —Saturated aqueous solution of picric acid . .	75 parts
Formalin (40 per cent) . . . . .	25 parts
Glacial acetic acid . . . . .	5 parts

There is little danger of overfixation and the fluid penetrates rapidly.

McClung and others have found that the addition of urea enhanced the preservation of finest cellular details. Allen's modification is recommended:

Saturated aqueous solution of picric acid . . . . .	75 parts
Formalin (40 per cent) C.P. . . . .	15 parts
Glacial acetic acid . . . . .	10 parts
Urea . . . . .	1 part

The addition of chromic acid, 1 part, to Allen's formula is also recommended. It must be added just before using for the chromic acid is reduced by the formol with the development of a greenish color. Other discolorations or precipitates are due to impurities in the reagents used and render the fluid useless.

## WASHING

Tissue need not be washed in water after formalin or alcohol fixation. Tissues fixed in solutions containing chromium salts should be washed for 24 hours in running water. Those fixed in picric acid solution will be macerated if left too long in water; washing should therefore be brief (several hours).

## METHODS FOR THE DEHYDRATION OF TISSUES

**Ethyl Alcohol.**—This is the most common dehydrating agent. It should be used in ascending strength, 70 per cent, 80 per cent, 95 per cent, and absolute alcohol. Where one wishes to reduce shrinkage to a minimum, still weaker solutions must be employed. Regardless of the manner of dehydration with alcohol, there will be shrinkage but it will be less with the slower and more gradual process. Iso-butyl alcohol has been used

with slightly less shrinkage than that caused by ethyl alcohol. It is toxic and its vapors may cause headache.

**Dioxane (Diethyl Oxide).**—This is a colorless liquid which boils at  $101^{\circ}$  C. It is a solid below  $8^{\circ}$  C. It is highly volatile, mixes with all proportions of water and alcohol. It dissolves paraffin slightly when cold and quite readily when heated. According to Baird and Bucher while it does not completely meet the requirements of a perfect dehydrating agent it is definitely superior to any substances used at present. Tissues remain soft and do not become brittle. There is little or no shrinkage depending upon the tissue itself, and there is no distortion. In Bucher's and Blakely's experience, dioxane has enhanced rather than detracted from the effect of some of the commonly used stains. No special preparation of the tissue is necessary except for colloid or colloid-like substances which have been fixed in formalin. These must be washed in running water for 12 or 24 hours. Under other circumstances, tissues are treated as for dehydration by alcohol. It is most important to remove all paraffin from the section before staining, for even traces of paraffin will interfere. Dioxane is inflammable and no attempt should be made to redistill it. There must be no open flame in the room where it is employed. Its vapors have a slight anesthetic effect. Therefore, all containers must be kept covered or tightly stoppered.

Bucher and Blakely have designed a small perforated cup of porcelain which rests in a stender dish (93 mm. in height and 62 mm. in diameter) upon glass supports so that the tissue in the cup lies at about the middle of the fluid volume.\* Dishes are filled  $\frac{3}{4}$  with the reagent. Their technic is as follows:

1. Fixed tissue not more than 4 mm. thick is placed in a mixture of 1 part distilled water and 3 parts by volume of dioxane (unrefined, commercial). This step lessens shrinkage and may be omitted. One hour is sufficient time in this mixture.

2. The second dish contains pure dioxane. Anhydrous calcium chloride is placed in the bottom of the dish to about the depth of 1 cm. It prolongs the usefulness of the reagent for after about 2 weeks it may be removed, the dioxane filtered and used again. Two to 3 hours is sufficient to complete dehydration. A longer time does not harm the tissue.

3. Infiltration after dioxane does not differ greatly from the procedure commonly followed. The tissue is supported above the bottom of the paraffin container by copper wire baskets (about the size and shape of the porcelain cups). As the dioxane is volatile, several changes of paraffin are not necessary. The tissue has a tendency to fall out of ribbons if all the dioxane is not removed. Infiltration for a period of 8 to 12 hours seems to accomplish complete removal and thorough infiltration. We recommend paraffin having a melting point of  $56^{\circ}$  to  $58^{\circ}$  C.

The method of staining is that commonly used. Alcohol instead of dioxane is employed as a dehydrating agent for sections. The authors mentioned above prefer Harris's hematoxylin stain followed by immersion in 2 per cent aqueous solution of sodium hyposulphite for 15 to 30 seconds. The section is of course to be washed in water after each step. Xylol is used as a clearing agent before mounting in balsam.

**Acetone.**—While acetone is suitable for small fragments of tissue it is not generally recommended for it produces shrinkage and shattering of tissue. Small fragments may be dehydrated by 2 or 3 changes of acetone of  $\frac{1}{2}$  to 1 hour each.

\* May be purchased from A. H. Thomas Co., Philadelphia.

### METHODS FOR THE CLEARING OF TISSUES

Since ethyl alcohol is not miscible with paraffin or celloidin, the embedding substances to be employed, it is necessary to find some substance which will remove the alcohol from the tissue and be in turn miscible with paraffin. Iso-butyl alcohol or dioxane require no clearing agents for these substances will mix with paraffin. There are 3 commonly used clearing agents, namely, cedar-wood oil, benzol and chloroform. Some, but not all of these, render the tissue translucent, hence the name of the procedure is inappropriate.

**Cedar-Wood Oil.**—Tissues should be placed after dehydration in a mixture of equal parts of cedar-wood oil and absolute alcohol and then in pure cedar-wood oil. From 2 to 12 hours are required for each depending upon the nature of the tissue. The tissue is translucent when completely "cleared." The oil should be washed from it with chloroform for several minutes before placing it in paraffin. Several changes of paraffin are required to remove the last traces of oil. Some workers prefer to place the tissue in a mixture of equal parts of oil and paraffin and then into 2 changes of paraffin for the best results.

**Benzol.**—Benzol may be used for small pieces of tissue that clear quickly. It hardens too much when treatment is prolonged. One-half to 1 hour is required for fragments 2 to 3 mm. in diameter. When the action is complete the tissue is quite translucent.

**Chloroform.**—Tissues are placed in pure chloroform after dehydration. After 4 to 8 hours they may be transferred to a mixture of equal parts of chloroform and paraffin where they remain for 4 to 8 hours.

### PARAFFIN METHODS FOR THE INFILTRATION AND EMBEDDING OF TISSUES

**Infiltration.**—Infiltration is designed to give the tissue rigidity so that it may be cut in extremely thin sections, and to hold together its fragments. Paraffin and celloidin are commonly employed. Paraffin is the most popular infiltrating agent. That having a melting point of 56° to 58° C. is chosen. In very cold weather, that having a melting point of 52° C. is more desirable. It is recommended that a large supply be kept on hand, for new paraffin tends to crystallize, a change which renders section cutting impossible. Infiltration is obviously carried on in some form of oven that maintains a constant temperature slightly above the melting point of the grade used (1 to 2 degrees). It should never be permitted to go above this point lest the tissues be damaged. Mixtures of paraffin and cedar-wood oil or chloroform should be placed on top of but not in the oven. Some prefer 2 changes of paraffin. The first having a melting point slightly lower than the second or final bath. Tissues of the thickness recommended (3 to 5 mm.) are usually completely infiltrated at the end of 4 or 6 hours. However, they are frequently left in the paraffin oven over night. Some workers have found the addition of rubber improves the quality of the sections obtained. The following formula is recommended (Baird):

Prepare a saturated solution of crude rubber in paraffin (m.p. 53° to 55° C.).

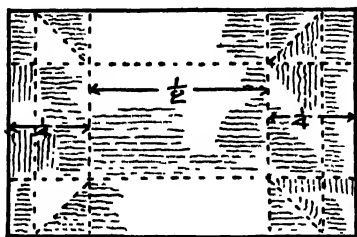


This is the stock rubber-paraffin, with which the embedding medium is prepared.

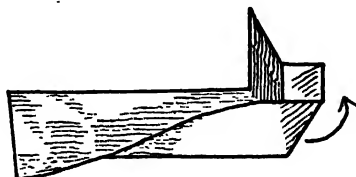
Stock rubber paraffin (winter months) . . . . .	160 grams
(summer months) . . . . .	140 grams
Paraffin (m.p. 53° to 55° C.) . . . . .	720 grams
Bayberry wax . . . . .	80 grams
Aniline oil . . . . .	5 cc.

It may be used as plain paraffin.

**Embedding.**—Tissues may be embedded in paraffin on wooden blocks, in paper cups or in Petri dishes. Oak blocks 1 x 1 x 1.5 inches are the most suitable. Wrap a piece of paper 2½ x 6 inches, around the block so that a cup is formed on one end (Fig. 340). A rubber band will hold the paper in place. Pour some hot melted paraffin



1. Crease on dotted lines.



2. Fold up one end and one side.  
Fold projecting flap against the end.



3. Repeat with adjoining corner.



4. Fold over and crease the project-  
ing end-flap to lock the corner joints.



FIG. 340.—THE METHOD OF FOLDING THE PAPER CUPS

(From Gradwohl, *Clinical Laboratory Methods and Diagnosis*, C. V. Mosby Co., St. Louis.)

into the cup and set the tissue in it keeping in mind the manner in which it is desired that the tissue appear in the sections. For example a piece of intestine must be placed on its edge so that the microtome knife will cut cross sections of its wall. Fill the cup with paraffin until the tissue is well covered. Set the block aside until the surface solidifies. Then plunge the entire block into ice water or stand it next to ice in the refrigerator until it has completely hardened. If the metal disk is used to carry the paraffin block in the microtome, embedding must be done in paper cups or in a Petri dish. The block prepared by any method must be neatly trimmed and the edges beveled so that the surface is a true rectangle. The sections will separate more readily if the corners of the block are cut away. The tissue should be well surrounded by paraffin on all sides. Chill the block on ice before placing it in the microtome. Routine

sections should be cut 6 to 8 microns thick. If the tissue has been properly infiltrated it will form long ribbons as sections are cut. Transfer the ribbon to a dish of warm water (40° to 45° C.) with the aid of a camel's hair brush and fine forceps. If the water is of the correct temperature (40° to 45° C.), the sections will flatten out completely and they may be separated by poking gently at the nick that has formed between the sections as the result of cutting the corners of the block. Float a section upon a slide which has been smeared with Mayer's albumin:

This adhesive material may be prepared by mixing equal parts of egg white and glycerin. Beat the mixture thoroughly until it flows evenly like thin syrup. Filter and add 1 per cent sodium salicylate as a preservative.

Place a tiny drop upon a slide and smear evenly over the entire surface with the finger. The excess may be wiped away with palm of the hand. Only a very thin film is desired. After the sections have been properly located, permit them to dry well protected from dust for several hours or better overnight.

## SUMMARY OF METHODS FOR INFILTRATING AND EMBEDDING IN PARAFFIN

**Slow Method.**—1. Fixation of tissue not more than 5 mm. thick.

10 per cent formalin.....	12 to 24 hours or,
Zenker's fluid .....	12 to 24 hours or,
Bouin's fluid .....	6 to 12 hours
Wash in running water.....	12 to 24 hours after Zenker's.

2. Dehydration.

Ethyl alcohol 70 per cent.....	12 hours
Ethyl alcohol 80 per cent.....	12 hours
Ethyl alcohol 95 per cent.....	12 hours
Ethyl alcohol 100 per cent.....	12 hours

3. Clearing.

Ethyl alcohol 100 per cent and	
Cedar-wood oil equal parts.....	12 hours
Pure cedar wood oil.....	12 hours

4. Infiltration.

Cedar-wood oil and	
Paraffin equal parts .....	12 hours (Place on top of oven)
Paraffin 1 .....	8 hours
Paraffin 2 .....	8 hours

5. Orientation and embedding.

Cool block quickly.

**Rapid Method.**—Tissue must not be more than 3 mm. thick.

1. Fix in formol-alcohol 1 to 4 hours.
2. Dehydrate in acetone, 3 changes each  $\frac{1}{2}$  hour.
3. Clear in benzol for  $\frac{1}{2}$  hour or until translucent.
4. Infiltrate with paraffin for 2 to 4 hours or overnight.
5. Orient and embed as in method No. 1.

**Dioxane Method (Bucher and Blakely).**—1. Fixation as in method No. 1.

2. Dehydrate in dioxane mixture for 1 hour:

(a) Dioxane ..... 2 parts

Water ..... 1 part

(b) Pure dioxane for 2 to 3 hours.

3. Infiltrate with paraffin 8 to 12 hours.

4. Orient, embed as in method No. 1.

## CELLOIDIN METHODS FOR THE INFILTRATION AND EMBEDDING OF TISSUES

It was once believed that paraffin could only be used for small sections of tissue, but Wainwright and others have succeeded in preparing beautiful sections of whole breasts and other organs embedded in paraffin. There are tissues, however, which are damaged or hardened by the heat of the paraffin oven so that some embedding material is desired that may be employed at room temperature. This requirement is met by trinitrocellulose, sold for this purpose under the name of Celloidin or Parloidin. Some workers have found commercial guncotton as sold by the Hercules Powder Co. to be just as satisfactory and considerably cheaper. It may be purchased in 5 pound tins and of course must be carefully stored, far removed from any source of heat. *The cans must never be opened with a steel instrument* lest a spark be generated and the guncotton ignited. Celloidin is used for embedding in 2 or more solutions. The first is thin, just half the strength of the stock or thick celloidin. Thick celloidin is prepared by dissolving 30 grams of dried material in 200 cc. of a mixture of equal parts of absolute alcohol and ether (Mallory). One must carefully dry the chips of celloidin, since they are preserved in water. Parloidin is now obtainable in dry glass bottles. It is customary to place the wet chips upon a piece of absorbent paper for 24 hours far removed from any heat source or flame. They should be protected from dust. While large pieces of tissue such as whole brain sections may be embedded in celloidin, tissues cut better if they measure not more than 2 cms. on a side and 5 mm. thick. The trimmed pieces are fixed and dehydrated in the usual manner. After dehydration in absolute alcohol, they are transferred to a mixture of absolute alcohol and ether where they remain for 24 hours. They may then be transferred to thin celloidin. Here they must remain for at least 24 hours or better, for several days. Finally the pieces are soaked in the thick solution for 1 or more days. The pieces should then be grasped with a pair of forceps carrying as much of the celloidin with them as possible and oriented on a vulcanized fiber block. Block and tissue are immediately plunged into chloroform where they are held submerged for 1 or 2 hours. After this they may be stored in 80 per cent alcohol. Some prefer to place the tissue in moderately thick celloidin in a dish fitted with a tight cover. After sufficient soaking 1 or more days, the cover is lifted for a few hours permitting a film of celloidin to form on the surface. The lid is replaced and overnight the vapor of ether dissolves the surface film with consequent concentration of the mass of celloidin including that which has infiltrated the tissue. This process is repeated until the mixture acquires a moderately firm consistency. Blocks may now be cut out with a knife, cemented to the fiber blocks with thick celloidin, plunged into chloroform as before and finally stored in 80 per cent alcohol. Sections may be cut on any microtome though the sliding type is preferred.

The knife must be set at an angle of 45 degrees or more so that nearly all the cutting edge passes through the tissue. The block and the knife must be kept wet with 80 per cent alcohol. The sections may be transferred to water or 80 per cent alcohol where they remain until stained. They may be stained upon the slide or in staining dishes. Nearly all methods are applicable without removal of the celloidin. If it becomes necessary, oil of cloves or the alcohol and ether mixture may be employed to dissolve the celloidin. If celloidin sections are carefully blotted and the wrinkles ironed out, they will ordinarily adhere quite firmly to the slide. They must never be permitted to become dry during the staining process.

**Celloidin Method (Custer).—**1. Fix tissue in formalin or Zenker's solution.

2. Wash in running water for 24 hours.

3. Dehydrate in alcohol.

65 per cent (with iodine for Zenker's fixed tissue) . . . . .	12 hours
85 per cent . . . . .	12 hours
90 per cent . . . . .	24 hours
95 per cent . . . . .	24 hours
100 per cent . . . . .	24 hours
100 per cent (with anhydrous copper sulphate as a desiccator) . . . . .	24 hours
100 per cent alcohol and ether, equal parts (with copper sulphate) . . . . .	24 hours

4. Infiltrate. Pass through 5 solutions of celloidin from very thin to very thick, each 2 days.

5. Place in a covered dish containing thick celloidin and control evaporation so that the celloidin hardens from within outward until it has the consistency of hard rubber.

6. Cut cubes and preserve in 80 per cent alcohol.

7. Dry cube and cement to fiber block with thick celloidin. Harden in 65 per cent alcohol.

8. Keep knife and block wet with 6 per cent alcohol while cutting. Very thick celloidin is made by dissolving about 22 grams of the chips in 100 cc. of alcohol and ether mixture.

## METHODS FOR THE STAINING OF TISSUES

The manner in which tissues or cells become stained when exposed to certain dyes is not at all clear. Once, it was supposed that the phenomenon was a chemical one, that basic substances would be stained by acid dyes and *vice versa*. At least it is not always so, for there is much to indicate that the process is purely physical, in which capillarity and osmosis account for the penetration of the dye. Adsorption may explain many of the phenomena of differential staining. Adsorption is a property possessed by a solid body, of attracting to itself, minute particles of matter from a surrounding fluid. These particles may exist as ions. The fact that the rate of adsorption may be influenced by the presence of other ions and that the reaction of the solution has a profound effect explains the phenomena of differential staining of various cellular elements, variation in the rate of staining with changes in the salt content of the

staining solution and the influence of the H-ion concentration upon color assumed by tissue when it is exposed to the action of both acid and basic dyes. Only the best available stains should be purchased for preparing solutions. For many years only the dyes of German manufacture could be relied upon and there are still some obtainable from Grüber that cannot be equaled. However, the dyes prepared by the National Aniline and Chemical Company, or Coleman and Bell in this country have given very satisfactory results. Whenever possible stains should be purchased which have been certified by the Commission on the Standardization of Biologic Stains of Geneva, New York.

Hematoxylin and eosin are the stains used routinely employed; they are prepared as follows:

**Hematoxylin (Delafield):**

Hematoxylin crystals .....	4 grams
Alcohol 95 per cent.....	25 cc.
Saturated solution of ammonium alum.....	400 cc.

A saturated solution of alum should be kept on hand. It is made by dissolving 180 grams of ammonium alum in 1000 cc. of water by the aid of heat. When the solution cools, some of the alum will crystallize, but the supernatant fluid will be saturated. Dissolve the hematoxylin in the alcohol and add it to the alum solution. Expose the solution to sunlight and air in an unstoppered bottle for 3 or 4 days. Filter and add:

Glycerin .....	100 cc.
Alcohol 95 per cent.....	100 cc.

Permit the solution to stand in the light until it is quite dark. It keeps well and its state of preservation may be determined by pouring a little into a beaker of water. A good stain develops a purple color while a stain that is poor or no longer effective causes a red solution. The action of hematoxylin depends on the presence of alum. Old solutions which stain too diffusely may be restored by the addition of alum to the stock. Precipitates are constantly forming; therefore, the solution must be filtered before it is diluted for use. It is the author's practice to dilute the stock with an equal volume of water before using.

**Hematoxylin (Harris):**

Hematoxylin .....	1 gram
Alcohol .....	10 cc.
Dissolve the hematoxylin in the alcohol.	
Alum (ammonium or potassium).....	20 grams
Distilled water .....	200 cc.

The addition of 8 cc. of glacial acetic acid increases nuclear staining.

The alum should be dissolved in the water by the aid of heat and then the alcoholic solution of hematoxylin added. Heat the mixture to the boiling point and add half a gram of mercuric oxide. As soon as the mixture develops a dark purple color, cool it quickly by plunging the vessel which contains it into cold water. It is ready for use when it has cooled. The solution stains well and may be kept for a long time. It is especially recommended for Zenker fixed tissues.

**Eosin.**—Eosin Y (yellowish) is one of the most valuable of plasma stains. It is labeled as water soluble but it is also soluble in alcohol. It is made as follows:

Eosin Y (85 per cent dye content).....	1.0 gm.
Ethyl alcohol (95 per cent).....	25.0 cc.
Water .....	75.0 cc.

Sections are stained from  $\frac{1}{2}$  to 1 minute.

**Eosin B** (bluish) is not a satisfactory stain. A bluish tint may be obtained by the addition of rose bengal or similar dye to eosin Y. Coleman and Bell offer such a product which is labeled "eosin bluish blend."

Paraffin sections are stained upon the slide. There are many types of staining jars available. The Coplin jar will accommodate 10 slides at a time for in it there are 5 grooves in each of which 2 slides may be placed back to back. Glass or metal racks carrying from 10 to 25 or more slides may be purchased when a great number of slides are to be stained at one time. The Coplin jar is the most popular; it is used for all of the reagents in the following steps:

1. Remove the paraffin by placing the slide in xylol for 5 minutes. A second bath in xylol is advocated for 3 minutes.

2. Wash in alcohol (95 per cent) for 5 minutes to remove the xylol.

3. If the tissue has been fixed in Zenker's fluid, place the slide in dilute tincture of iodine for 5 minutes to remove the precipitated mercuric salts.

This step is necessary only after Zenker's fixation. Some prefer to remove the mercuric salts by dissolving iodine in the first alcohol in the process of dehydration. This solution must have enough iodine dissolved in it to give a port wine color. It must be renewed as frequently as the iodine fades.

4. Wash in alcohol for 5 minutes to remove the iodine.

5. De-alcoholize the tissue in descending strengths of alcohol (80 per cent and 70 per cent) each for 3 minutes.

6. Wash in water.

7. Stain in hematoxylin for 5 minutes.

8. Wash in water and place in acid bath (1 per cent aqueous solution of hydrochloric acid) to remove the hematoxylin ( $\frac{1}{2}$  minute) from all but the nuclei. Control decolorization by frequent examination of the tissue with the microscope. The stain must remain in the nuclei.

9. Wash in water. If one drop of ammonium hydrate is added to a jar full of water, the stain will deepen quickly. The ammonia must be thoroughly removed by washing before the next step.

10. Allow the slide to stand in pure water for 5 minutes to deepen stain.

11. Stain in eosin (1 per cent aqueous, water soluble, yellowish eosin) for  $\frac{1}{2}$  to 1 minute.

12. Wash in water and then dehydrate in ascending strengths of alcohol—80 per cent, 95 per cent and absolute alcohol. Each solution for 3 minutes.

13. Clear in beechwood creosote or xylol for 5 minutes.

The selection must be translucent. If xylol is used and tissue is not thoroughly dehydrated, a milky fluid will be formed which can be removed by returning the slide to the alcohol bath.

14. Drain and wipe the clearing fluid from the slide around the sections. Place a

drop of Canada balsam on the tissue, cover with a coverglass. If the balsam does not immediately spread over the section without the inclusion of air bubbles under the cover, it is too cold or too thick. Gentle warming followed by gentle pressure will accomplish the desired result. If the balsam is quite thick, add a little xylol and mix until it has a thin syrupy consistency. It is important to add just the right sized drop of balsam. If too much is added, it will flow from beneath the cover and cause a sticky, dirty preparation. It is important also that not too much mounting media be used lest it interfere with the distinctness of the microscopic image, or prevent the use of the oil immersion lens. If too little balsam is used, the whole section will not be covered, air bubbles will form and the tissue will eventually dry out.

**Mallory's Phloxine and Methylene Blue Stain.**—This was formerly called the eosin methylene blue method until it was discovered that some of the staining qualities of this method were really due to the presence of phloxine in what was incorrectly sold as eosin. This mixture produces a sharp nuclear stain and reveals with marked differentiation the various structures in tissues. Tissues should be fixed in Zenker's fluid.

1. Stain paraffin sections in a 5 per cent aqueous solution of phloxine (dye content about 80 per cent) for 20 minutes or longer.

2. Wash away the excess phloxine with water.

3. Stain for 30 minutes in borax methylene blue solution diluted in 10 parts of water:

Methylene blue (medicinal 90 per cent dye content)	1 gram
Borax .....	1 gram
Water .....	100 cc.

Pour this solution on and off the section several times.

4. Wash in water.

5. Differentiate and dehydrate in 95 per cent alcohol in a dish to which has been added a few drops of 10 per cent collophonium. The section must be kept in constant motion so that decolorization will be uniform. Control the result under the microscope. When the pink color has returned to the section and the nuclei are still blue, complete dehydration quickly with absolute alcohol.

6. Clear in xylol.

7. Mount in balsam.

#### **Phosphotungstic Acid Hematoxylin (Mallory):**

Hematein ammonium .....	0.1 gram
Water .....	100.0 cc.
Phosphotungstic acid crystals (Merck)....	2.0 grams

Dissolve the hematein in a little water with the aid of heat. After it is cool add it to the phosphotungstic acid dissolved in about 80 cc. of water. Dilute finally to 100 cc. The solution should be permitted to stand for several weeks to ripen before it is used. However, ripening may be hastened by the addition of 5 cc. of a 0.25 per cent solution of potassium permanganate. This permits immediate use of the stain. Hematoxylin may be substituted for the hematein ammonium. If this substitution is made, add 10 cc. of the permanganate solution to ripen the mixture.

Zenker fixed tissue is required for this stain. Kernohan has obtained satisfactory results with formalin fixed tissue after it has been especially treated in Weigert's mordants.

1. Wash the tissue for several hours in running water or in dilute ammonia for a short time.

2. Fix 4 days in Weigert's primary mordant for myelin sheaths:

Potassium bichromate .....	5.0 grams
Chromium fluoride (furochrom).....	2.0 grams
Water .....	100.0 cc.

3. Fix 2 days in Weigert's secondary mordant for myelin sheaths:

Copper acetate .....	5.0 grams
Chromium fluoride .....	2.5 grams
Acetic acid (36 per cent).....	5.0 cc.
Water .....	100.0 cc.
Formalin .....	10.0 cc.

Nuclei, fibroglia, myoglia, neuroglia fibrils, fibrin and contractile elements of striated muscle are stained blue; collagen and ground substances of bone, varying shades of yellow or brownish red. Elastic fibrils develop a purplish tint. Tissues are run through the preliminary steps as for the routine staining method until they are brought into the first wash water after the removal of paraffin:

1. Place in 0.25 per cent aqueous solution of potassium permanganate for 5 to 10 minutes.

2. Wash in water.

3. Oxalic acid, 5 per cent aqueous solution for 10 to 20 minutes.

4. Wash thoroughly in water.

5. Stain in phosphotungstic acid hematoxylin for 12 to 24 hours.

6. Transfer to 95 per cent alcohol and then to absolute alcohol. Dehydrate quickly for the red stain is easily extracted.

7. Clear in xylol and mount in balsam.

**Aniline Blue Collagen Stain (Mallory).**—This stain is useful for the study of collagen, fibrin, fibroglia, muscle and amyloid. Collagen fibrils and reticulum of connective tissue, amyloid and mucus are stained blue; nuclei, cytoplasm, fibroglia fibrils, neuroglia fibrils, axis cylinders and fibrin red; red blood cells and myelin sheaths yellow; elastic fibers, pale pink or yellow. The dye is prepared as follows:

Aniline blue soluble in water (certified).....	0.5 gram
Orange (certified) G. 80 to 85 per cent dye content.....	2.0 grams
Phosphotungstic acid .....	1.0 gram
Water .....	100.0 cc.

Prepare tissues as for the routine stain to the first wash water.

1. Stain sections in 0.25 per cent aqueous solution of acid fuchsin for 30 minutes. Drain and immediately:

2. Stain in the aniline blue solution for 1 to 24 hours or longer. Staining in paraffin oven for 1 hour gives as good a result as over night in the cold.

3. Wash in several changes of 95 per cent alcohol and then in absolute alcohol.

4. Clear in xylol.

5. Mount in balsam.

If the tissue is first stained lightly in Delafield's hematoxylin, nuclei and smooth muscles stain brownish color.



**Azocarmine Modification (Haidenhain) of Mallory's Aniline Blue as Employed by McGregor for the Study of Renal Glomerulus.**—In general the results are best following Zenker or Helly fixation. However, tissues fixed in 10 per cent formalin may be treated as follows:

(a) For paraffin sections of formalin-fixed tissue:

1. Ammonia (40 drops to 10 cc. water) . . . . . 1 hour
2. Running water . . . . . 1 hour
3. Zenker's or Helly's fluid . . . . . 1 hour
4. Running water . . . . . 1 hour

(b) For blocks of formalin-fixed tissue:

1. Ammonia (40 drops to 100 cc. of water); 2 days in paraffin oven at 40° C.
2. Running water . . . . . 24 hours
3. Zenker's fluid . . . . . 5 hours
- or
- Helly's fluid . . . . . 12 hours
4. Running water . . . . . 12 hours

1. One per cent azocarmine G (1 gm. in 100 cc. water, heat, cool, filter at room temperature and add 1 cc. glacial acetic acid). Stain 30 to 40 minutes in the paraffin oven at 57° C.

2. Wash in water.

3. Differentiate in aniline alcohol (1 cc. aniline oil in 100 cc. 95 per cent alcohol). Watch under the microscope until the nuclei are red and the cytoplasm pale pink. This step requires from 1 to 3 minutes, depending on the thickness of the sections.

4. Remove aniline with acid alcohol, about 1 minute.

5. Three hours in 5 per cent phosphotungstic acid.

6. Wash quickly in water.

7. Stain 3 to 6 hours in the following:

Aniline blue . . . . .	0.5 gram
Orange G . . . . .	2.0 grams
Glacial acetic acid . . . . .	8.0 cc.
Distilled water . . . . .	100.0 cc.

Boil, cool and filter. Dilute  $\frac{1}{2}$  with water.

8. Wash in water.

9. Differentiate in absolute alcohol, watching under the microscope.

10. Xylol.

11. Balsam.

Nuclei appear orange-red, cytoplasm pink, connective tissue and reticulum blue, fibrin red.

**Van Gieson's Stain.**—This is a differential stain for collagen which assumes a red color. All other tissues are stained yellow. Best results are obtained with material fixed in chromium salts (Zenker's fluid). The stain is prepared as follows:

- 1 per cent aqueous solution of acid fuchsin . . . . . 5 cc.
- Saturated aqueous solution of picric acid . . . . . 100 cc.

Solutions must always be tested upon tissues containing collagen. If the collagen does not stain a definite red a little more of the acid fuchsin must be added. The solution may lose its differential staining qualities if exposed to the light for a long period. Therefore it should be tested from time to time. Sections are stained in the following manner:

1. Stain deeply with alum hematoxylin as in the routine method.
2. Wash in water and stain with Van Gieson's mixture 3 to 5 minutes.
3. Dehydrate in 95 per cent alcohol.
4. Clear in oil of origanum (pure).
5. Mount in balsam.

**Unna's Alkaline Methylene Blue Solution.**—This solution was recommended by Unna for staining plasma cells:

Methylene blue (90 per cent dye content) . . . .	1 gram
Potassium carbonate (Merck) . . . . .	1 gram
Water . . . . .	100 cc.

Its chief value is in the preparation of Unna's polychrome methylene blue. The alkaline solution is permitted to ripen for months when, as the result of oxidation, methyl violet and methyl red are formed. The fully ripened solution may be obtained from Grübler. Dilute 1 to 10 before staining.

**Terry's Neutralized Polychrome Methylene Blue.**—This is a modification of Goodpasture's formula. Three aqueous stock solutions are prepared:

1. 12 per cent anhydrous potassium carbonate.
2. 1.0 per cent methylene blue (medicinal—90 per cent dye content).
3. 10 per cent (by volume) acetic acid.

One cc. of solution 3 is titrated with solution 1 using phenolphthalein as the indicator. The amount of solution 1 required to exactly neutralize 1 cc. of solution 3 at the boiling temperature is introduced into a 100 cc. cylinder and enough of solution 2 is added to bring the final volume to 100 cc. This mixture is then divided into 4 equal parts of 25 cc. Each part is placed in a 1-ounce bottle and the 4 bottles in a basin of cold water which is gradually heated to boiling. The time of boiling is noted and at the end of 15 minutes 1 bottle is removed, the others at the end of 20, 25, and 30 minutes respectively. They are permitted to cool slowly and to each is added 0.25 cc. of solution 3. The solutions are now ready for use though they will improve upon standing. Filtration is usually not necessary and should not be done for 1 or 2 days. If one determines by trial which of the 4 bottles contains the best stain, the next lot may be boiled for that length of time which gave the best results.

**Verhoeff's Elastic Tissue Stain.**—Formalin or Zenker's fixative may be used in preparation for this stain but the best results are obtained with the latter. Tissues are not to be treated with iodine for the staining solution itself will remove the precipitated mercuric salts. The solution must be freshly prepared for it does not keep longer than one month:

Hematoxylin crystals . . . . .	1 gram
Absolute alcohol . . . . .	20 cc.

Dissolve by the aid of heat, filter and add in the order given:

Ferric chloride (10 per cent aqueous solution) . . .	8 cc.
--	-------

Lugol's solution (iodine, 2 parts; potassium iodide,  
4 parts; water, 100 parts)..... 8 cc.

- 1. Stain sections until perfectly black (15 minutes).
- 2. Differentiate in 2 per cent aqueous solution of ferric chloride. This step requires but a few seconds. Control it by examining the section with the microscope. If carried too far the section may be restained.
- 3. Wash in water and then in 95 per cent alcohol to remove the iodine.
- 4. Wash in water again and stain for 1 minute with eosin.
- 5. Dehydrate, clear and mount as in routine method.

By this method, elastic tissue is stained black but connective tissue, fibroglia, myoglia, and neuroglia are stained red by the eosin.

**Best's Carmine Stain for Glycogen.**—Glycogen is quite soluble in water; therefore, when it is to be demonstrated, aqueous fixatives cannot be used. Absolute alcohol is the best fixative and celloidin the best infiltrating agent. Of course the sections cannot be placed in water but may be placed in alcohol until stained. The staining mixture is prepared as follows:

Carmine ..... 2 grams  
Potassium carbonate ..... 1 gram  
Potassium chloride ..... 5 grams  
Water ..... 60 cc.

Boil gently for several minutes but do not overheat. Cool the solution and add:

Ammonium hydrate ..... 20 cc.

The mixture will keep for several months in tightly stoppered bottles. It should be filtered and dilute before using in the following manner:

Carmine solution ..... 2 cc.  
Ammonium hydrate ..... 3 cc.  
Methyl alcohol ..... 3 cc.

- 1. Stain sections deeply with hematoxylin.
- 2. Decolorize with acid alcohol if the stain is too diffuse.
- 3. Wash quickly but thoroughly in water.
- 4. Stain with the diluted carmine solution for 5 minutes.
- 5. Differentiate in the following mixture:

Absolute ethyl alcohol ..... 80 cc.  
Methyl alcohol ..... 40 cc.  
Water ..... 100 cc.

- 6. Wash in 80 per cent; then in absolute alcohol.
  - 7. Clear and mount in balsam.
- By this method nuclei are stained blue and glycogen a bright red.

**Amyloid Stain.**—Fresh or formalin-fixed frozen sections are best suited for the demonstration of amyloid. Celloidin sections do not stain with the aniline dyes unless the celloidin is removed.

1. Stain frozen sections in 1 per cent aqueous solution of methyl violet for 5 minutes.

2. Wash in 1 per cent aqueous solution of acetic acid.

3. Wash thoroughly in water to remove the acid.

4. Mount in glycerin jelly.

Amyloid is stained red, other tissues are blue violet.

The stain is not permanent.

Iodine green may also be used in a 0.3 per cent aqueous solution. Amyloid is stained a violet red.

**Stain for Fat.**—It is obvious that unless the fat is fixed by osmic acid solution, it will be dissolved in the dehydrating agents. Therefore where fat is to be demonstrated, the tissue must be cut by the freezing method. Formalin fixation may be employed. Scarlet R., better called Sudan 4, is the stain of choice. Prepare a saturated solution in a mixture of equal parts of 70 per cent ethyl alcohol and acetone. The dye may be added to the solvent in a 2-ounce bottle until a small excess accumulates in the container on standing. After it has completely settled, pipet a small amount of the solution into the stender dish. Care must be taken not to carry any of the sediment into the staining jar for it will precipitate upon the section. When not in use, the solution must be kept in tightly stoppered bottles for the acetone evaporates quickly.

1. Stain sections for 5 minutes in the saturated solution in a stender dish.

2. Transfer with a section lifter to 70 per cent alcohol for an instant.

3. Wash in water and counter stain in hematoxylin.

4. Wash in water.

5. Mount in glycerin jelly.

These sections are not permanent, though they may be kept for months if the cover-glass is rimmed with asphalt paint. Fat is stained red, nuclei blue.

**Iron Containing Pigments.**—Such pigments as hemosiderin may be demonstrated in fresh or formalin fixed tissue cut by the freezing method. Tissues may be embedded in celloidin. The reagent is prepared as follows:

SOLUTION A.—2 per cent aqueous solution of potassium ferrocyanide.

SOLUTION B.—1 per cent aqueous solution of hydrochloric acid.

These 2 solutions are preserved separately and mixed when ready for use. Add 1 part of solution A to 3 parts of solution B.

1. Stain sections in the mixed reagent for 20 to 30 minutes.

2. Wash in distilled water.

3. The section may now be stained with hematoxylin and eosin.

4. Wash in distilled water.

5. Mount in glycerin.

## METHOD OF SILVER IMPREGNATION

A silver impregnating technic that is applicable to formalin or Zenker fixed tissues, that will reveal the finer fibrils as well as the other elements of a tissue, particularly tumors, is of great value to the histologist. The method given below was designed particularly to demonstrate the fibrils of tumors of the melanoma group (Foot and Foot). In making use of this stain the table should be consulted and that variant

selected which will give the desired results. If one desires a selective stain of reticulum, one of the first 3 variants should be employed; the last 3 are unsatisfactory for they impregnate collagen and reticulum exactly alike, magenta or reddish. According to Foot and Foot, the second and fifth are the best for general use. The fourth or the sixth give less intense impregnations than the fifth. For delicate effects, with little disturbance of the cytoplasmic background, the first variant should be chosen; the fourth if more cytoplasmic detail, color variety and plasticity are desired. Those variants employing the tannic acid mordant will give more colorful pictures than those where it is omitted. Variant 5 demonstrates muscle striations well. Variant 2 reveals beautifully the reticulum of the liver sinusoids, lymphatic reticulum and muscle sheaths.

The methods do not impregnate either brain or spinal chord with sufficient contrast.

**Fixation.**—The best fixative is neutral 10 per cent formalin in which blocks cut thin enough to insure complete penetration of the fluid should remain 24 hours at least; longer if possible. If Bouin's fluid is used, the results are comparable to those obtained in the Laidlaw-Bouin method; the nuclei will be unimpregnated, the cytoplasm impregnated in the cast of epithelial cells, and mesoblastic cells will be unstained. The resulting pictures are more colorful than those obtained by the Laidlaw procedure.

The method gives very good results if Zenker-fixed tissues are used. They should be fixed for 24 hours and washed in running water for another 24 hours. After embedding and sectioning, the mercuric chloride should be removed from the sections with the usual alcoholic iodine solution, and this in turn removed with very weak (1 per cent or less) aqueous sodium thiosulphate. This must then be washed out thoroughly. The oxidation-reduction steps, in which the potassium permanganate and oxalic acid are used, should be omitted as they produce effects similar to Bouin's fixation. The presence of chromium salts makes no material difference in the subsequent impregnation, except to enhance the impregnation of nervous tissue. On the whole, formalin fixation gives more colorful results and is, on this account, to be preferred. This does not, however, imply that Zenker's fixation is to be eschewed—quite the contrary; it gives very striking pictures in all instances and is well suited to the method.

**Embedding.**—The ordinary routine method of paraffin embedding is used after dehydration of the tissue in ascending percentages of alcohol and chloroform.

**Preliminary Treatment.**—This is essential in the case of all the variants. The sections are de-paraffinized in 2 changes of xylol and absolute alcohol and are then treated from 1 to 24 hours with a mixture of 2 parts pure pyridin to 1 part of pure glycerol. This bath keeps well and may be used repeatedly for many weeks. The sections are transferred directly from this to 2 changes of 95 per cent alcohol, washed in tap water and placed in distilled water.

**Impregnating Fluid.**—This is a simple silver diamino hydroxide solution. It is used in all the variants, at full concentration in the first 3, at half strength in the last 3. To 10 cc. of 1.2 per cent silver nitrate solution in distilled water, strong ammonia is added dropwise until the resulting brown precipitate is just dissolved; 10 cc. of 3.2 per cent pure sodium hydroxide solution in distilled water is added and the reprecipitated silver hydroxide again just dissolved by the addition of a few more drops of ammonia. The solution is then made up to a 100 cc. with distilled water that has been heated to about 50° C. Sections are impregnated in this in a closed staining

# COLOR VARIATIONS IN TISSUES STAINED WITH SILVER BY 6 VARIANTS

Tissue	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	Variant 6
Nuclei	Brown	Magenta, slightly brownish	Dull magenta-brown	Brown or black	Black	Sharp brown red-dish or black
Epidermal cytoplasm	Slate brown to brown	Slate violet to magenta	Rose slate, brownish to magenta	Slate brown to fuscous	Violet to violet-brown	Slate blue to slate brown
Glandular cytoplasm	Slate brown	Slate brown to magenta	Magenta-gray	Pinkish gray to brown	Pinkish gray to violet	Violet brown
Erythrocytes	Brown	Magenta	Dark brown to black	Reddish brown	Violet-brown	Brown to seal brown
Collagenous fibers	Lilac to light magenta	Deep magenta to violet	Dull magenta	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Reticular fibers	Black	Black	Dark magenta to black	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Endoneurial fibers Meissner's nevus cells	Red to black	Magenta to black	Magenta to violet or black	Red to black	Magenta to black, finest often carmine	Brick red
Skeletal muscle fibers	Slate brown striae black	Magenta to dark red, striae red to brown	Slate pink, striae red to brown	Pinkish brown striae black	Violet, striae deep magenta	Violet to black; striae indistinct, too intense
Cardiac muscle fibers	Gray	Magenta-gray	Slate pink	Gray, striae blackish	Violet, striae magenta	Violet, striae magenta
Smooth muscle fibers	Gray	Magenta	Rose-gray	Pinkish to brownish gray	Violet	Slate violet
Myelin sheaths	Black	Black	Black	Pinkish red to magenta	Magenta to scarlet-magenta	Brick red
Nerve trunks	Pink to red	Magenta	Magenta	Brownish pink, epineurium darker	Magenta, epineurium darker	Brick red, epineurium grayish
Melanin	Black	Blue-black	Black	Black	Blue-black	Black

box in the incubator at 37° C., or the paraffin oven at 55° C. for 1 hour in case of variants 1, 2, and 3, and for 10 minutes in half-strength solution (5 cc. silver nitrate, 5 cc. sodium hydroxide) in that of the other 3 variants.

Silver diamino carbonate may be used interchangeably with, and in the place of, the hydroxide; it often gives superior results, particularly in those variants in which the tannate mordant is used. It is made up at full strength in all cases; to 10 cc. of 10.2 per cent silver nitrate add strong ammonia drop by drop until the precipitate is dissolved. Then add 10 cc. of 3.1 per cent sodium carbonate in distilled water, instead of the hydroxide. There is no reprecipitation upon adding the carbonate, as the hydrogen ion concentration remains unchanged, and further ammonia is therefore unnecessary. The solution is used in exactly the same manner as the hydroxide.

**Reducing Fluid.**—The developer is a mixture of 1 cc. of strong neutral formalin (40 per cent formaldehyde); 1 per cent sodium carbonate in distilled water 3 cc., and distilled water to make 100 cc. Three minutes completes the reduction.

**Toning and Fixing.**—The toning bath is a 1:500 solution of Merck's "acid brown" gold chloride in distilled water. The fixing fluid is the usual 5 per cent aqueous solution of sodium thiosulphate ("hypo").

**VARIANT 1.**—The sections are taken from distilled water, impregnated for 1 hour in the impregnating fluid, washed in 2 changes of distilled water and reduced in the developer for 3 minutes or so. They are then washed in tap water and toned for 3 or more minutes in the gold bath, washed and fixed in "hypo" solution for 3 or more minutes, after which they are washed, dehydrated in ascending percentages of alcohol, cleared in xylol and mounted in Canada balsam.

**VARIANT 2.**—This is similar to the preceding formula, except that the Laidlaw oxalic acid (5 per cent) bath is intercalated between the toning and fixing baths, and the fact that toning, redevelopment and fixing are all lengthened to 10 minutes each, to correspond with Laidlaw's directions.

**VARIANT 3.**—In the variant, formalin-soda replaces the oxalic acid procedure of its predecessor. It is made up exactly as before (formalin 1 cc., 1 per cent sodium carbonate 3 cc., distilled water to 100 cc.). Used developer should not be employed; it should be made up freshly each time. The treatment with gold, formalin and "hypo" solution is the same as in variant 2. (Instead of soda-formalin solution, a solution of 0.5 per cent oxalic acid in 5 per cent neutral formalin has been found to give better results and avoids the danger of precipitates.)

**VARIANT 4.**—In the following 3 variants a tannic acid mordant is used made up as follows: pure tannic acid 0.2 gram; ammonium bromide 3.5 grams; strong neutral formalin 5 cc.; distilled water to make 500 cc.

The sections are mordanted for 15 minutes in the tannic acid bath heated to 50° C. in the incubator or paraffin oven. They are then treated for ½ to 1 minute with 100 cc. of distilled water to which has been added 3 to 5 drops of strong ammonia. This is the "stop" solution. They are then washed for about 2 minutes in distilled water. The impregnation with silver is complete at the end of 15 minutes instead of 1 hour, as in the preceding variants. After impregnation the sections are washed in distilled water, developed, toned and fixed as in variant 1.

**VARIANT 5.**—Proceeding as in variant 4, the method changes as soon as the toning bath is reached, to correspond with variant 2, lengthening the time to 10 minutes and using the 5 per cent oxalic acid-gold developed in exactly the same manner.

**VARIANT 6.**—This resembles variant 5 in every particular except one, formalin-soda developer replaces the oxalic acid bath, as in variant 3.

The formalin-oxalic acid intensifier may be used here, as in variant 3.

**Summary of Steps in the Variant.**—1. Neutral formalin or Zenker's fixation.

2. Paraffin embedding.

3. Pyridin-glycerol pretreatment for 1 to 24 hours.

4. In variants 4, 5, and 6; tannic acid mordant for 15 minutes followed by "stop" solution of ammonia for 30 seconds.

5. (a) Variants 1, 2 and 3; impregnation in warm silver diamino hydroxide for 1 hour.

(b) Variants 4, 5 and 6; impregnation in this bath at half-strength for 10 minutes.

6. Reduction of silver in formalin-soda developer for 3 minutes.

7. Toning in 1:500 gold chloride in variants 1 and 4 for 3 minutes; other variants for 10 minutes.

8. Reduction of gold in variants 2 and 5 with 5 per cent oxalic acid; variants 3 and 6 with formalin-soda; in either case for 10 minutes.

9. Fixing in 5 per cent thiosulphate in variants 1 and 4 for 3 minutes; other variants for 10 minutes.

*Note:* Thorough washes are indicated between all steps, distilled water being required until the sections have been reduced in step 6; after that, tap water is employed throughout.

## METHODS FOR THE PREPARATION OF SECTIONS OF BONE

Formerly bone studies were made upon extremely thin disks prepared by grinding. The same methods were applied to the study of teeth. The information gained by the study of such sections is of value chiefly to the student of normal histology rather than the pathologist. Morrell has described an especially hard knife capable of cutting fresh untreated bone in section 6 to 12 microns thick. With a technic so simple, as compared with the tedious and time consuming methods of grinding, perhaps more will be learned concerning the microscopic structure of bones in health and in disease. The present methods (Jaffe) deal chiefly with decalcified bone. There are a number of decalcifying agents none of which are entirely satisfactory because they cause swelling of cells or prevent subsequent differential staining.

Neutral formalin in a 10 per cent aqueous solution is the best fixative, for swelling is less after its use than after any other fixative. Either before or after fixation the bone should be cut into slices not more than 2 or 3 millimeters thick. A thin fine hack saw blade or better still, a jeweler's saw will serve satisfactorily for small pieces but for large pieces a band saw is best. The decalcifying agent must be used in large volumes. It must be frequently agitated and changed several times during the process. It is best to decalcify in the incubator (37° C.). Decalcification must not be prolonged; its progress may be determined by sticking a fine needle into the specimen, never by bending it. The tissue must be promptly removed and thoroughly rid of the decalcifying agent when the action is complete.

**Decalcifying Agents.**—Zenker's fluid because of its acetic acid content will remove small amounts of calcium during the process of fixation but it has little effect



upon bone fragments. Sodium citrate in 20 per cent solution has been recommended for many have noticed that calcareous specimens preserved in fluids containing sodium citrate become soft. Müller's fluid, like Zenker's, will decalcify if the tissue is not too dense. It is frequently used for fetal bone. It requires weeks to accomplish this purpose and fibrillar staining may be impossible after such prolonged action of bichromate.

*Nitric Acid.*—A 5 per cent solution of this acid seems to be the choice of most pathologists. It is perhaps the least harmful of all decalcifying agents if used properly. A short exposure to an acid of this strength is less harmful than prolonged exposure to a weaker solution. The aqueous solution is more effective than the alcoholic. Small bubbles of gas collect about the tissue and retard the action of the acid unless the container is frequently shaken or placed in a mechanical shaker. If the bone is of the compact variety, the acid solution must be changed several times. Decalcification should be complete within 18 to 24 hours. A longer exposure to the acid will surely impair the staining qualities. After the action of the acid is complete, transfer the tissue to a 5 per cent solution of sodium sulphate for 24 hours. During this period the sulphate solution must be frequently changed until it no longer becomes acid to litmus but remains neutral. Wash the tissue in running water for 24 hours after which it may be dehydrated and embedded.

Some prefer to add to the acid solution some substance that will counteract the swelling effect. Phloroglucin is recommended by Mallory. The solution is prepared as follows:

Phloroglucin .....	1 gram
Nitric acid .....	10 cc.

The solution should be made in a 500 cc. or liter flask for considerable heat is generated, and if a smaller vessel such as a cylinder or small flask is used the mixture will be expelled by the gaseous vapors generated. *Always keep the mouth of the flask pointed in such a direction that if the fluid is ejected it will do no harm.* After solution is complete and the fluid has cooled slightly it may be diluted with 100 cc. of 10 per cent nitric acid. The resulting solution is about 20 per cent nitric acid. If a weaker solution is desired, use less acid in preparation but do not dilute the final mixture for phloroglucin is not usually effective in protecting the tissue in less than 1 per cent solution.

*Hydrochloric Acid.*—Hydrochloric acid is used in 5 per cent solution in 10 per cent sodium chloride. After decalcification the tissue should be placed in 10 per cent sodium chloride to which small amounts of lithium carbonate are added until the solution becomes neutral. After 48 hours in salt solution the tissue is washed in running water for 24 hours. This is the best method for the preservation of fibrillar structure.

*Formic Acid.*—In mixture with sodium citrate this acid has been recommended but in the hands of the author it has not been as satisfactory as nitric acid, and it is much more costly.

*Waggoner's Solution.*—This method is usually satisfactory for large or dense sections of bone. The specimen may be left in the solution for 1 to 2 months with no damage to the staining properties of the nuclei.

Fixation may be in either 10 per cent formalin or Helly's solution but acetic-Zenker solution should not be used.

After fixation, the specimen should be washed free of the odor of formalin or over night in running water if Helly's solution has been used.

Waggoner's solution (which keeps for several months) is prepared as follows using Merck's 85 per cent to 90 per cent C.P. formic acid with a specific gravity of 1.2 and a molecular weight of 46.02:

Formic acid (85 per cent to 90 per cent) . . . . .	50 cc.
Distilled water . . . . .	35 cc.
Mix and add to following solution:	
Sodium citrate (Merck C.P.) . . . . .	17 gms.
Warm distilled water . . . . .	85 cc.

When the bone has been decalcified, wash in tap water to remove the odor of formic acid, embed by the paraffin or celloidin methods, remove the mercuric precipitate if fixation has been in Helly's fluid and stain as desired.

**Dehydration, Embedding and Staining.**—After thorough washing in running water for 24 hours, the tissue is ready for dehydration. Decalcified bone may be embedded in paraffin or celloidin. Celloidin is the better agent, because paraffin embedded bone acquires considerable hardness. The first 6 or 8 sections cut should be discarded, because the surface of the block of tissue is certain to have been lacerated by the saw or fragments of bone dust. Decalcified bone is not easily stained. One may employ a strong Delafield's hematoxylin and slightly overstain and then permit the sections to stand in water overnight. The Hansen Bock technic yields a sharp differential stain. The following solutions are required:

I. Hematoxylin . . . . .	1 gram
Absolute alcohol . . . . .	10 cc.
II. Potassium alum . . . . .	20 grams
Distilled water . . . . .	200 cc.
III. Potassium permanganate . . . . .	1 gram
Distilled water . . . . .	16 cc.
IV. Glycerin C.P. . . . .	50 cc.
Glacial acetic acid . . . . .	50 cc.
V. Eosin Y . . . . .	4 grams
95 per cent alcohol . . . . .	1000 cc.

(a) Mix solutions I and II and then add 3 cc. of solution III. Boil for 1 minute; filter when cool. This stain must be freshly prepared each time it is to be used. Stain unmounted celloidin sections in this mixture for 2 to 18 hours. (Paraffin sections may be washed in xylol to remove the paraffin and then washed with alcohol and water, after which they may be handled as frozen sections and stained as above.)

(b) Differentiate the stained sections in solution IV. Differentiation may be controlled by frequently floating the section on a slide and examining it with the microscope. As soon as a satisfactory result is obtained, the section should be plunged into water and washed for 1 hour. Differentiation usually requires from 5 to 20 minutes.

(c) Counter stain in solution V for 5 minutes.

(d) Dehydrate as in the routine methods, and mount in balsam.

**PREPARATION AND STAINING OF BONE MARROW SECTIONS**

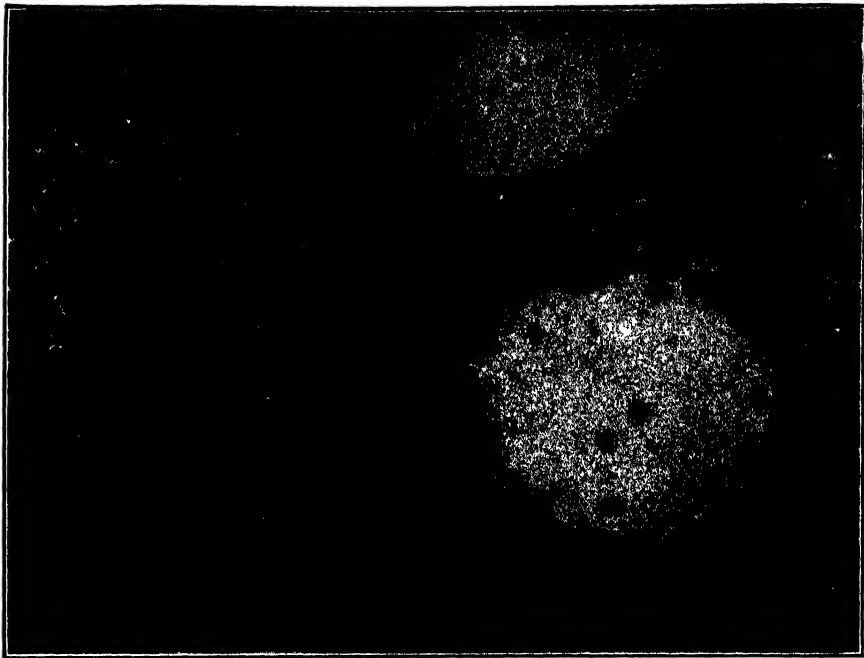
In the preparation of bone marrow it is important to avoid dense bone that will require decalcification. When bone marrow biopsies are studied, it is well to divide the specimen into 2 parts. The first, the button of the bony cortex which must be decalcified, and the second the soft marrow portion which at the most contains a few spicules and does not require a special decalcifying agent. Both pieces may be fixed in



**FIG. 341.—THE METHOD OF EXPRESSING MARROW FROM A RIB**

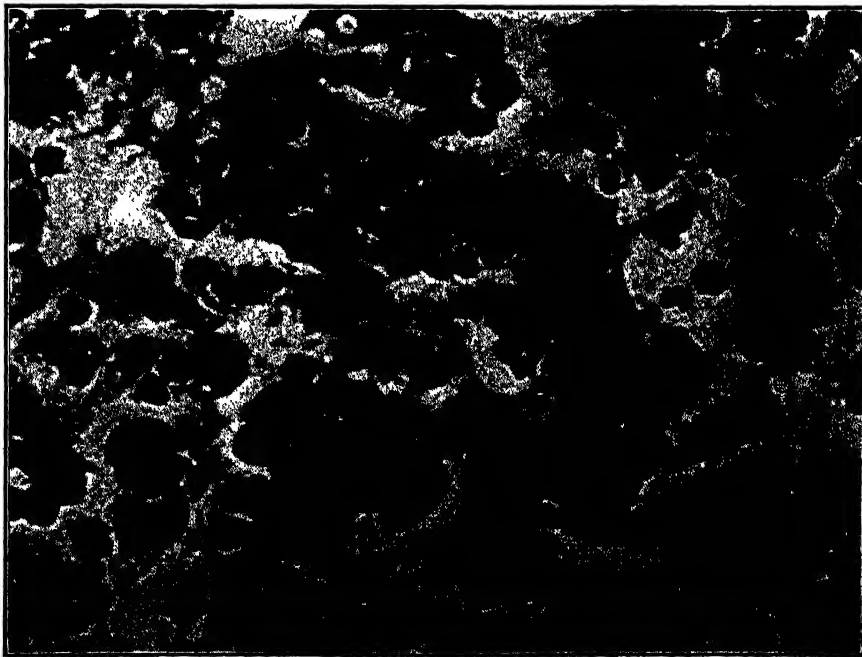
The rib is thoroughly cleaned of fascia, and then, hand-over-hand, two pair of pliers squeeze the marrow from one end of the rib to the other. It emerges as shown in photograph, in thick drops, which are permitted to fall directly into the fixing solution. (Suggested by Dr. Max Lederer, Brooklyn Jewish Hospital.)

Helly's fluid. The bony button must be decalcified but the soft marrow may be washed, dehydrated and embedded in paraffin. Rib marrow may be squeezed from the rib as in Figure 341. Two pair of pliers worked hand over hand from one end of the rib to the



**FIG. 342.—NORMAL STERNAL BONE MARROW (young adult)**

About 60% cellular, the remainder fat; myelocyte: erythroblast ratio from 2:1 to 6:1. A focus of erythropoiesis is seen in the center, nearly all cells being of the late erythroblast and normoblast stages. The background of large, pale cells are myelocytes, metamyelocytes and a few scattered reticulum cells.



**FIG. 343.—PERNICIOUS ANEMIA (RELAPSE) (sternal biopsy)**

Marrow is solidly cellular and composed of a disorderly mass of megaloblasts, later forms of the red cell series being sparse and imperfect. The myeloerythroid ratio is reversed.

other will force out sufficient marrow for study. The droplets may be received directly into Helly's fluid. Sections of vertebrae may be decalcified in Muller's fluid if time is not a factor. When speed is necessary, frozen sections may be made of the fixed spongy portion provided the tissue be impregnated with 12 to 15 per cent glycerin gelatin. This requires about 5 hours at 37° C. The gelatin is allowed to cool and harden and then a block may be cut out carrying the tissue with it. Further hardening may be accomplished by soaking in 10 per cent formalin. The block should be trimmed and well washed for at least an hour before staining.

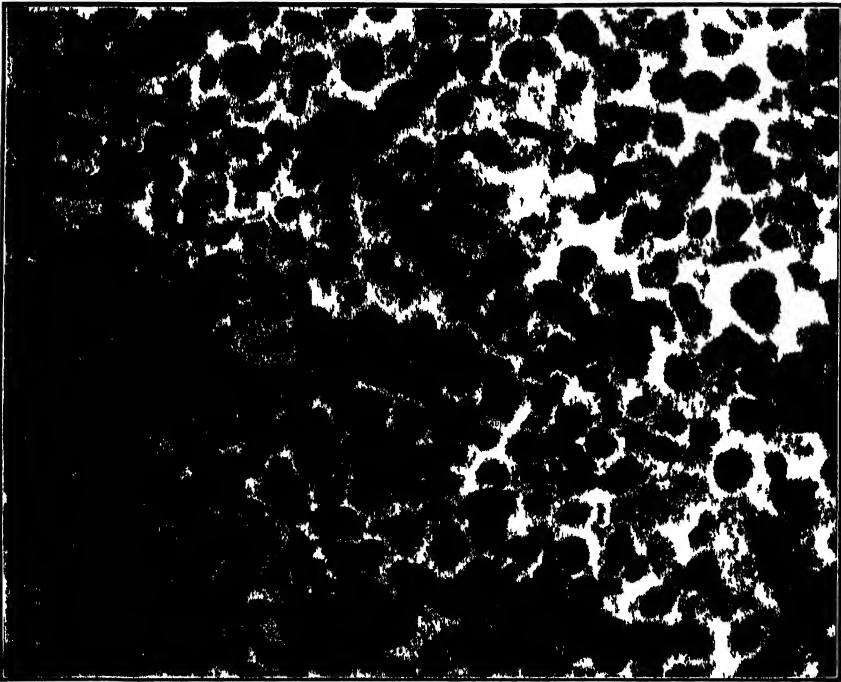


FIG 344—IDIOPATHIC HYPOCHROMIC ANEMIA (sternal biopsy)

Marrow is totally cellular and erythropoiesis is extremely active; red cell formation is of the normoblastic type although there is a scattering of megaloblasts present. Hemoglobin content of the later nucleated red forms is exceedingly sparse, normoblasts often appearing as naked nuclei with a colorless halo.

Sections may be stained by the routine hematoxylin and eosin technic or by the methylene blue phloxine technic of Mallory. According to Schmorl the Ellerman modification of the May-Grünwald technic is the stain of choice. The method is carried out as follows:

1. Fixation in Helly's fluid containing 10 per cent of formalin. The tissue should be obtained promptly after death.
2. Wash in running water 24 hours.
3. Dehydrate, clear and embed in paraffin, cut sections 5 microns thick.
4. After fixation on a slide and removal of paraffin, wash in water and remove

the excess water with absorbent paper. Then place in the following mixture for 15 minutes:

Eosin 1 per cent aqueous solution..... 5 cc.  
Neutral formalin ..... 0.25 cc.

5. Wash in warm (45° C.) distilled water.
6. Stain in May-Grünwald solution (Grübler) diluted with equal amounts of water for 30 minutes.
7. Wash 5 to 10 minutes in distilled water. Blot dry with absorbent paper.
8. Differentiate in 100 per cent alcohol 2 to 4 minutes by dropping the alcohol upon the preparation until no more color comes away and the section assumes a red tone.
9. Clear in xylol and mount in balsam.

Nuclear structure of the cells of the hematopoietic system and especially the neutrophils are well stained by this method. (Figs. 342, 343, 344 and 345.)

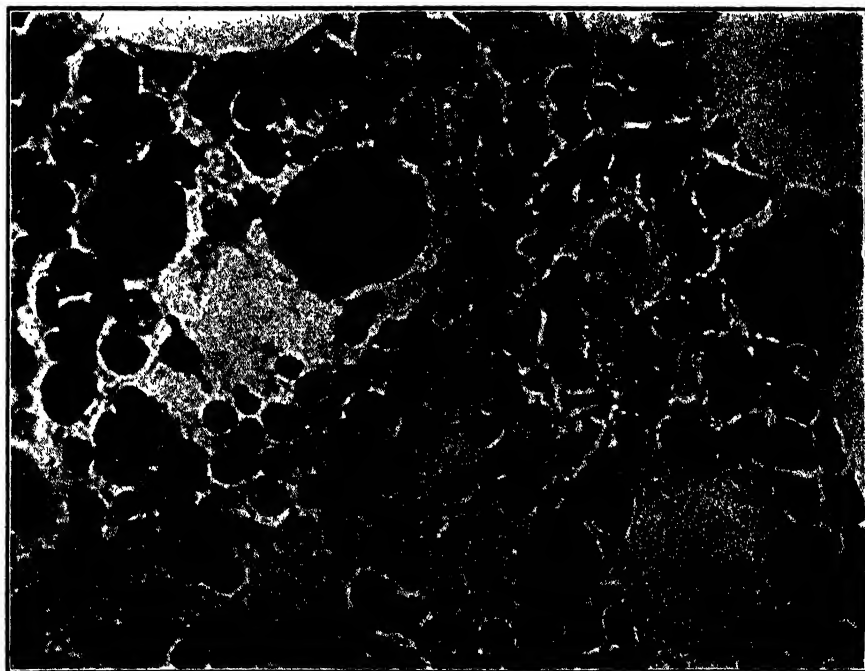


FIG. 345.—HEMORRHAGIC ANEMIA (sternal biopsy)

Cellularity is increased over the normal for the age, the majority of cells belonging to the erythropoietic series and found in the later stages of maturation. The three large cells are megakaryocytes.

### CUSTER'S MODIFICATION OF AZURE II—EOSIN METHOD FOR BONE MARROW \*

This method if carefully followed yields very satisfactory sections. The various cells reveal a most striking differential stain. Experience and practice are required and the slightest variation in technic may result in failure.

1. Fix the biopsy material in formol-Zenker (Helly's) solution.
2. Wash gently in running water for 1 hour.
3. Decalcify in Waggoner's formic-citrate mixture over night if necessary (until the bony button is soft to the prick of a needle).
4. Wash gently in running water for 1 hour.
5. Dehydrate in ascending strengths of alcohol.
6. Clear in chloroform.
7. Infiltrate through chloroform and paraffin and embed in paraffin.
8. Cut sections 3 to 4 microns thick and mount on slides in the usual manner.
9. The sections are carried through xylol, absolute 95 per cent alcohol as in the routine technic. The precipitated mercury is removed from the sections by immersing for several minutes in a 2 per cent solution of iodine in 95 per cent alcohol, followed by immersing in 80 per cent alcohol, tap water, 5 per cent hypo (sodium hyposulphite or thiosulphate solution), tap water, and finally in 3 changes of distilled water. All traces of alcohol must be removed from the tissue. The stain is prepared as follows:

Stock solution I—Eosin Y (Coleman and Bell) 1-1000 aqueous solution.

Stock solution II—Azure II (Nat'l. Aniline and Chem. Co.) 1-1000 aqueous solution.

10. When ready for staining mix in glassware reserved for this purpose:

Distilled water .....	80 cc.
Solution I .....	20 cc.
Solution II .....	10 cc.

11. Filter through cotton.

It is well to prepare this mixture at the close of the day, immerse the slides in a covered jar and stain until the next morning. But the preparations must not be left in the stain too long. The next morning they should be immediately differentiated in two changes of 95 per cent ethyl alcohol. Control differentiation by examination with the low power objective of the microscope. Wash the preparations in 2 changes of absolute alcohol and clear in 2 changes of xylol. Mount in balsam or damar.

If the tissue is very bloody, it is advisable to use but 5 cc. of the aqueous eosin solution (solution I), but no other departure from the technic is permissible.

### MASSON TRICHROME STAINING METHODS

Foot has quite properly pointed out that too many laboratories limit their histologic technic to hematoxylin and eosin staining and that even the more common special stains for revealing the less evident tissue structure are not employed as frequently as indicated. The beautiful and striking differential staining accomplished

\*Courtesy of Miss D. Broun, Philadelphia Gen. Hosp.

by Masson has been considered too complicated for the average hospital laboratory technician. Foot, however, has succeeded in simplifying Masson's technic. Obviously the results are not as perfect as those obtained by the original methods but, as Foot comments, "the more usual procedure will afford (with Masson's stain) preparations that are vastly better than the usual laboratory products."

**Fixation.** Masson recommends a Bouin's solution composed of commercial formalin 10 parts, 2 per cent aqueous solution of trichloroacetic acid 2 parts and water 30 parts. Picric acid crystals are added in excess, with frequent shaking until a saturated solution is obtained. This requires about 3 days. He recommends 3 days' fixation but 1 will suffice if the blocks are not more than 5 mm. thick. The tissue is not washed but transferred to 80 per cent alcohol. He also uses a bichromate solution consisting of 90 parts of 3 per cent potassium bichromate and 10 parts of commercial formalin. The latter is added freshly each time the fluid is employed. Foot also finds that 10 per cent neutral formalin or 10 per cent neutral formalin in 95 per cent ethyl alcohol, or even Zenker's fluid, give excellent results though the color scheme will not be the same as by Masson's prescribed technic. The bichromate solutions require the tissue to be washed for 24 hours in running water. The sections subsequently must be immersed in weak alcoholic iodine solution followed by weak aqueous solution of sodium hyposulphite (sodium thiosulphate).

**Dehydration and clearing.**—These are accomplished as described in the text, using ascending strength of ethyl alcohol (8 to 12 hours for each) and chloroform. Infiltration in paraffin preceded by immersion in chloroform saturated with paraffin, is carried out as in the usual technic. Two changes of paraffin are used over a period of 5 hours. Tissues are embedded in paraffin in paper cups and are sectioned in the usual manner. Thin sections of not more than 5 microns are required. There must be no furrows or irregularities in thickness for the intense stain will over stain thick or ridgy sections.

**Fastening the Sections to the Slide.**—Masson uses a gelatin solution and a tedious technic. Mayer's albumin may be employed in the usual manner although sections will wash off in the heated stains unless great care is exercised. When the sections are thoroughly dried they are prepared for staining by removing the paraffin with xylol, washing with alcohol and finally in running water.

**Staining.**—There are a number of stain combinations yielding various color combinations. Foot has found the iron hematoxylin-Ponceau-Acid fuchsin light green combination most valuable and employs it as a routine stain.

**Nuclear stain.**—1. Sections are first mordanted in the following solution for 5 minutes in the oven at 45° to 50° C.:

Iron alum .....	5 gms.
Distilled water .....	100 cc.

2. Wash in tap water for several minutes.
3. Stain in Regaud's hematoxylin prepared as follows:

Hematoxylin .....	1 gm.
Ethyl alcohol—95 per cent.....	10 cc.
Glycerine .....	10 cc.
Distilled water .....	80 cc.



Dissolve the hematoxylin in hot distilled water, cool and add the alcohol and glycerine. The stain may be used at once.

4. Staining requires 5 minutes or more in the oven at 45° to 50° C.
5. The sections will be now uniformly black. Wash in 95 per cent alcohol.
6. Differentiate in the following solution:

Saturated solution of picric acid in 95 per cent ethyl alcohol. . . 2 parts  
Ethyl alcohol 95 per cent. . . . . 1 part

Decolorization takes place rapidly. Control the progress under the microscope and as soon as the nuclei alone remain colored, immerse the slide in running water for 15 minutes. If, after washing, the background remains gray, immerse in alcohol and complete the picric differentiation. Wash again in running water.

*Cytoplasmic stain.*—1. The stain is prepared as follows:

Ponceau dexylidine (Krall; Eimer and Amend). . . . . 1 gm.  
Glacial acetic acid . . . . . 1 cc.  
Distilled water . . . . . 100 cc.

2. After washing in the water the sections are stained in this solution for 5 minutes, rinsed with distilled water, immersed in a 1 per cent aqueous solution of phosphomolybdic acid solution for 5 minutes and thoroughly washed in distilled water.

3. A wider range of red colors may be obtained varying from vermillion to ruby if two parts of the above ponceau solution are mixed with 1 part of the following:

Acid fuchsin . . . . . 1 gm.  
Acetic acid glacial . . . . . 1 cc.  
Distilled water . . . . . 100 cc.

The procedure as outlined above is followed.

4. If either of the above stains are diluted with 10 volumes of 1 per cent acetic acid and the staining time lengthened to 1 hour, greater delicacy and precision of staining will result.

*Connective Tissue Stain.*—1. After washing in distilled water, immerse the slide in the following stain:

Light green (Krall; Eimer and Amend). . . . . 2 gms.  
Glacial acetic acid . . . . . 1 cc.  
Distilled water . . . . . 100 cc.  
Add the dye to the acid water.

2. Stain for 5 minutes. The rapidity of staining varies with different samples of green.

3. Wash the preparation with 1 per cent aqueous solution acetic acid for 2 minutes; dehydrate; clear in xylol and mount in balsam.

Nuclei will appear blue black; cytoplasm various shades of red; mucus and collagen green.

The technician is advised to consult Masson's original article for other stain combinations.

### **CYTOLOGIC STUDY OF VARIOUS BODY FLUIDS WITH ESPECIAL REFERENCE TO TUMOR CELLS**

Frequently pleural, peritoneal, spinal and other fluids are sent to the laboratory with the request that they be examined for tumor cells. It is the custom of many to attempt to stain smears of sediment with methylene blue or some other stain. The results are so unsatisfactory that even if recognizable tumor cells are present, they would not be revealed by this method unless they were present in large clumps. The author conceived the idea of concentrating the cell content first by centrifuging 30 cc. or more of the fluid if available. The supernatant fluid is poured off with the exception of the last 1 or 2 cc. Ten cc. of Helly's fluid is added and the tube with its contents again centrifuged. The whole is allowed to stand for 2 to 8 hours when the sediment may be dislodged as a solid button, washed in water and carried through the usual technic of paraffin embedding. If there is abundant sediment a large round bottom tube may be used which will produce a broad disk of solidified sediment. If the sediment is scant a regular centrifuge tube should be employed.

A similar procedure may be used when splenic puncture or sternal puncture yields only a bloody fluid. Needle biopsies of tumors likewise may yield fragments too tiny to be handled individually. Where salt solution washings carry the cells to be studied or whenever the fluid is known to be poor in proteins, it is well to pour off all the supernatant fluids after the first centrifuging and to add 1 to 2 cc. of clear serum before adding the Helly's fluid.

By this method, not only are the cells concentrated, but they are seen as one customarily sees tumor cells. Frequently the cells obtained in pleural or peritoneal fluids of malignant disease may be found to compare amazingly well with the cells of the tumor seen in paraffin sections.

### **TECHNIC OF ASPIRATION BIOPSY**

Aspiration biopsy has become a popular helpful procedure in the diagnosis of tumors when the conventional form is undesirable or impossible. Negative information obtained by this method must not be accepted, for the failure to obtain diagnostic tissue is greater than by the more inclusive conventional technic.

1. The collection of material is a surgical procedure to be performed only by some one with surgical experience. An 18-gauge needle of suitable length attached to a 20 cc. syringe, is plunged into the tissue after anesthetizing the skin until the tip of the needle is in the area from which biopsy is desired. Suction is formed by forcefully drawing out the plunger of the syringe and at the same time revolving the syringe and needle with the idea of the cutting edge of the needle removing a core of tissue which is then sucked into the needle by the negative pressure in the syringe. A few short insertions and withdrawals, repeating the revolving motion, may be performed to further aid in dislocating a small fragment of tissue. After withdrawal of the needle, the area may be covered for a few days with cotton impregnated with colloidion.

2. Aspirate a little salt solution into the syringe to wash tissue fragments out of the needle. Then transfer the fragments and salt solution to a centrifuge tube. Centrifuge 10 minutes at about 2400 r.p.m. Pour off all but 1 cc. of the supernatant

fluid. Add 1 cc. of serum, fill the tube with Helly's fluid. Centrifuge again to pack the cells. Allow the tube to stand several hours.

3. Remove the button of sediment, breaking the tube rather than the button. Wash in water 1 hour and proceed as with any other tissue. Because of the small size of the button, 4 to 5 hours in each step up to paraffin is sufficient. It is a good plan to leave the tissue in paraffin over night, before blocking out the button in half, and orient so that the section will show the various levels of depth. In other words, the plane of the section should be in the long axis of the tube in which the material was centrifuged.

4. Sections are cut and stained as for any other paraffin embedded tissue. Fragments of tissue large enough to permit recognition are commonly obtained from neoplasms.

### **METHODS FOR EXAMINATION OF STERNAL BONE MARROW**

The technic for obtaining sternal bone marrow has been described on page 953.

1. The needle is removed from the syringe so that the latter may be filled with air, then re-attach the needle and force the marrow in its lumen upon a grease free slide. A good size drop will usually accumulate. By touching this drop as if it were on a punctured finger, 2 or 3 films may be prepared as for leukocyte differential counts. The remainder is allowed to clot and the clot scraped into a small tube of Helly's fluid. It may then be prepared as a small fragment of tissue and embedded in paraffin. Stained sections of this fragment will give the general histological structure of marrow.

2. The films may be stained with Wright's stain. The author prefers Pappenheim's modification of the May Grünwald technic employed as follows:

(a) Air dried films are covered with May Grünwald stain (concentrated) for 3 minutes.

(b) Distilled water in amount equal to the volume of stain on the slide and well mixed with a pipet is added and allowed to remain 1 minute.

(c) The slide is flushed with water, drained and covered Giemsa stain (1:30 dilution) for 20 minutes.

(d) Flush with water and stand the slide on end to drain until dry. Do not blot.

Excellent cellular detail is revealed by this stain so that a fairly accurate differential count may be made.

This procedure may be repeated so that the progress of a blood dyscrasia may be followed and the effect of treatment observed. It may be employed on children but should not be attempted without experience on adults and a thorough knowledge of the anatomy of the sternum of the child. Children under the age of 1 year have very little sternal marrow arranged in small foci—usually opposite the interspaces—so that it is impossible to obtain satisfactory material.

# METHODS FOR THE PREPARATION OF MUSEUM SPECIMENS

FRANK W. KONZELMANN

No matter how small an institution or its laboratory may be, there should be an attempt made to preserve good pathological specimens. Good museum technic is acquired only with practice (Figure 346). The main object is to mount a specimen in such a manner that all of its features may be apparent. Specimens in jars should show the original color and surface texture. Consistency cannot always be preserved. The various fixing agents harden the tissue. Soft tissues, that are frequently handled, soon fall to pieces; so while it is well to keep the more common lesions as "wet" specimens in large crocks, the uncommon ones should be mounted in suitable museum jars.

## SELECTION OF MATERIAL FOR THE PATHOLOGICAL MUSEUM

Representative material is obviously the most desirable for the medical museum is primarily a teaching unit and not a collection of curiosities. Material should be placed in a fixing solution as soon after removal from body as possible. Autopsy material must be fixed very shortly after death. Postmortem changes alter the appearance and consistency of organs; exposure to air is certain to affect the color.

**Fixation.**—Thorough fixation by a properly prepared fluid is necessary. Large organs like the lung and liver or brain do not fix well. The same is true of huge spleens from cases of leukemia or large tumors. It is far better to preserve slices of these organs which will show the pathology well. Such specimens cost less to prepare and are more easily handled. The basic principles of preparation are herein laid down. More detailed accounts may be obtained by consulting the *Journal of Technical Methods* issued by the International Association of Medical Museums.

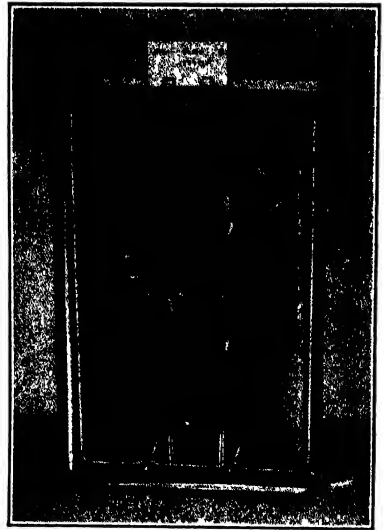


FIG. 346.—A HEART PROPERLY MOUNTED AND READY FOR THE MUSEUM

Note the position of the glass frame and its feet, one at the top and two at the bottom, which serve to keep the frame in the center of the jar. Note the position of the linen threads which support the specimen. They are efficient yet not unsightly. Note also the position of the label. (From Gradwohl, *Clinical Laboratory Methods and Diagnosis*, C. V. Mosby and Co., St. Louis, Mo.)

## KAISERLING METHOD

This is by far the oldest and most popular method in use today. It is also the most tedious. The organ should be suspended or laid in absorbent cotton in the position in which it will best reveal the lesion. Pieces of cotton should be inserted between opposing surfaces that touch so that the fluid may easily reach all parts. It must then be covered with an amount of fluid equal to about 10 times its volume.

Intestine requires about 24 hours for complete fixation. Thin organs, such as kidney, if cut through require 4 to 5 days. Spleen, liver, or lung may require weeks. It is better to cut slabs of the latter organ, not more than 4 cms. thick. Kaiserling fixing solution is prepared as follows:

Potassium acetate .....	170 grams
Potassium nitrate .....	90 grams
Formalin (neutral) .....	1600 cc.
Water .....	8000 cc.

Only the best chemicals should be used. Formalin may be neutralized with sodium hydroxide or marble dust.

*Color Restoration.*—After fixation as outlined above the specimen will appear to have lost much of its color. It must be thoroughly washed in running water for about 24 hours or until it has lost the odor of the formalin. It is now placed in 95 per cent alcohol. The color will gradually return. This step must be carefully watched, for after reaching its full intensity the color will fade again. Alcohol solutions lose their strength with use. The concentration of alcohol should never be permitted to fall below 80 per cent.

*Preservation.*—Wash the specimen again in running water and then place it in the preserving fluid prepared as follows:

Potassium acetate .....	1815 gms.
Glycerin .....	2000 cc.
Distilled water .....	10,000 cc.
Carbolic acid (preservative) .....	20 cc.

If the preserving fluid becomes cloudy it is best filtered with fine animal charcoal. Add one heaping tablespoonful of charcoal to each 2000 cc. and filter through filter paper in a large (2000 cc.) funnel. It may be necessary to pour the first filtrate back into the funnel.

### KLOTZ METHOD

This is less complicated and gives excellent results provided sufficient fluid is used. Solution I must be used in volumes 10 times as great as the organs being fixed and the solution must be changed when it becomes cloudy.

Solution I for fixation is prepared as follows:

Carlsbad salts (artificial) .....	1750 gms.
Chloral hydrate .....	1750 gms.
Formalin .....	1750 cc.
Water .....	35,000 cc.

About the same amount of time is required for fixations as with the Kaiserling method. After fixation, wash the specimen in running water for 24 hours. It is then placed in Klotz solution No. 2 prepared as follows:

Carlsbad salts (artificial) .....	875 gms.
Chloral hydrate .....	350 gms.
Formalin .....	175 cc.
Water .....	35,000 cc.

This is the final preserving solution. After several days or weeks, the specimen should be placed in fresh clear preservative and sealed in a suitable jar.

The formula of Klotz and MacLachlan for making artificial Carlsbad salts is as follows:

Sodium Sulphate .....	22 grams
Sodium Bicarbonate .....	20 grams
Sodium Chloride .....	18 grams
Potassium Nitrate .....	38 grams
Potassium Sulphate .....	2 grams

### SELECTION OF JARS

The square type jar is obtainable in many sizes. The cheaper domestic jars frequently show irregularities in the refraction of light giving a wavy effect. These jars should be rejected. Carefully selected domestic jars planed and polished on one surface are as satisfactory as the more expensive imported jars. Four sizes should be available. One that will conveniently hold a large heart ( $14 \times 12 \times 9$  cms.); a kidney jar ( $17 \times 12 \times 5$  cms.); intestine jar ( $32 \times 9 \times 5$  cms.) and a small specimen jar ( $16 \times 10 \times 5$  cms.)

Glass frame should be made to fit each jar snugly. For the jars listed above glass rods about 6 to 7 mm. diameter make the best frames. Soft glass is the more easily handled and just as serviceable as hard glass. The inside measurement of the jar should be carefully laid out as a rectangle on a sheet of asbestos. The rod should be marked with a wax pencil at the points where it is to be bent. Heat the glass with a blow torch at the first mark until it is red hot and soft. It may then be placed upon the asbestos and bent along the lines drawn. Each angle is bent until the rectangle is completed. Feet or cross bars must be fused on the frame so that it will remain in the desired position in the jar. These must be so placed that they support no weight but only prevent lateral motion of the frame. The entire frame must fit snugly within the jar. After the frame is completed it is well to heat a corner to red heat so that the frame may accommodate itself for the contraction which accompanies cooling.

Specimen should be fastened to frames with white linen thread. The thread must pass through fibrous connective tissue, as that which surrounds blood vessels, lest it pull or cut through. Small celluloid buttons may be used to prevent cutting by thread. One must always suspend the specimen with care and thought so that the lesion shows to the best advantage while the organ is seen in a natural anatomic position.

Labels must be attached to every specimen before mounting. The most satisfactory label is made by marking identification numbers upon linen tape with waterproof ink. The tape is then soaked in hot melted paraffin for 10 to 15 minutes. Such a label will last indefinitely.

After the specimen has been placed in the jar, it may be covered with the preserving fluid and then allowed to stand for several days, covered but not sealed. Air bubbles are certain to collect on the specimen or upon the sides of the jar. These may be dislodged with a small brush. The jar may now be sealed with asphalt cement. Trinidad lake asphalt has seemed the most satisfactory according to Muir and Judah. The cement must be heated on a sand bath until it has a liquid consistency. The cover of the jar should be pierced by a small hole to permit final filling of the jar

with fluid. The cover is heated in an oven until quite hot. The glass surfaces must be free of grease. Heat the top of the jar gently and then cover the edges with cement. Heat the cement on the jar edges and then place the cover in position and press it down firmly. While the cement is hot scrape off the excess. Allow the cement to harden and then fill the jar through the small hole in the cover. This hole may be plugged with a piece of cork or with sealing wax.

The specimen may now be completed with a suitable descriptive label which will indicate clinical and laboratory reference numbers, the source of the material, a short history of the case, microscopic findings and final diagnosis.

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